National Library of Canada

Acquisitions and Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada

Direction des acquisitions et des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

BILE TOLERANCE OF *LACTOBACILLUS* SPP.

By

Hanna M. Vaheri

©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Science

IN

Food Microbiology

Department of Agricultural, Food and Nutritional Science

EDMONTON, ALBERTA

SPRING 1996

National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-10760-4

Canada

UNIVERSITY OF ALBERTA

LIBRARY RELEASE FORM

NAME OF AUTHOR	**Hanna M. Vaheri**
TITLE OF THESIS	**Bile tolerance of *Lactobacillus* spp.**
DEGREE	**MASTER OF SCIENCE**
YEAR THIS DEGREE GRANTED	**1996**

Permission is hereby granted to the UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

(SIGNED) Hanna Vah

PERMENENT ADDRESS:

Munkkiniemen puistotie 7

00330 Helsinki

Finland

Dated Jan 26, 1996

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Bile tolerance of Lactobacillus spp.* submitted by *Hanna Mari Vaheri* in partial fulfillment of the requirements for the degree of *Master of Science* in *Food Microbiology*.

Michael E. Stiles

Dr. M.E. Stiles, Supervisor

Michael I. McBurney

Dr. M. McBurney
Committee Chair & Examiner

Lynn M. McMullen

Dr. L.M. McMullen, Committee Member

W. Page

Dr. W. Page, Committee Member

Date 15 December 1995

ABSTRACT

Lactobacillus spp. were used as a model to study alteration of bile tolerance in bacteria. Twelve strains of *Lactobacillus* spp. were screened for their sensitivity to bile. Three strains of *L. acidophilus* and *Lactobacillus* strain GG, that grew in 10% bile, were chosen for plasmid curing by growth in the presence of novobiocin at 45°C. No *Lactobacillus* strains were found that had lost their bile tolerance as a result of curing. Two variants of *L. acidophilus* ATCC 43121were cured of a 2.8 MDa plasmid. Because no loss of bile tolerance was observed in these strains, it was concluded that this plasmid did not mediate bile tolerance. The ability of bile sensitive *L. delbrueckii* subsp. *bulgaricus* strain J9 to adapt to bile was determined by exposure to increasing concentrations of bile. Two variants were obtained that had dramatically increased bile tolerance.

ACKNOWLEDGEMENTS

I would like to express my gratitude to:

My supervisor, Dr. M.E. Stiles, for his guidance and understanding during the course of my Masters program.

Dr. L. McMullen for her support during the last stages of my program.

M. Bennik and A. Poon for their technical assistance in the laboratory.

Dr. L. Quadri, Dr. M. van Belkum and Dr. R. W. Worobo for their assistance and advice in the field of molecular microbiology.

Friends and colleagues, especially L. Saucier and G. Alton, for their support and friendship.

My husband Lawrence for his incredible support and understanding.

This work was funded by the Dairy Bureau of Canada.

TABLE OF CONTENTS

1 INTRODUCTION 1

2 REVIEW OF THE LITERATURE 7

2.1 HISTORICAL REVIEW OF PROBIOTICS (with emphasis on the lactobacilli) 7

2.2 TAXONOMY OF THE *LACTOBACILLUS* SPP. 12

2.2.1 *L. delbrueckii* subsp. *bulgaricus* 13

2.2.2 *L. acidophilus* 14

2.2.3 *L. casei* 15

2.2.4 *Lactobacillus* GG 15

2.3 BILE IN THE HUMAN BODY 16

2.3.1 Bile metabolism in humans 16

2.3.2 Physiological concentration of bile acids 18

2.4 DECONJUGATION AND DEHYDROXYLATION OF BILE ACIDS BY BACTERIA 20

2.4.1 Deconjugation of bile by intestinal bacteria 20

2.4.2 Effect of bile deconjugation on the health of the host 23

2.5 BILE TOLERANCE OF BACTERIA 25

2.5.1 Antibacterial properties of bile acids 25

2.5.2 Effects of exposure of bacteria to bile 26

2.5.3 Effect of colony morphology on bile tolerance 29

2.6 USE OF BILE IN SELECTIVE BACTERIAL GROWTH MEDIA 31

2.7 SAFETY CONSIDERATIONS 33

3 MATERIALS AND METHODS 37

3.1 BACTERIAL CULTURES 37

3.2 CULTURE MEDIA 37

3.3 BILE TOLERANCE 39

3.4 SELECTION FOR INCREASED BILE TOLERANCE 40

3.5 PHYSIOLOGICAL TESTS ON EXPERIMENTAL CULTURES 41

3.5.1 Fermentation profiles 41

3.5.2 API identification strips 42

3.5.3 Coagulation of skim milk 43

3.6 PLASMID ISOLATION 43

3.6.1 Plasmid isolation according to a modified version of the method by Anderson and McKay (1983) 43

3.6.2 Small scale plasmid isolation according to O'Sullivan and Klaenhammer (1993) 46

3.6.3 Purification of plasmid DNA 47

3.6.4 Gel electrophoresis 48

3.7 PLASMID CURING 49

4 RESULTS 51

4.1 INITIAL SCREENING 51

4.1.1 Screening of strains of *Lactobacillus* spp. for bile tolerance 51

4.1.2 Characterization of the experimental strains by fermentation profiles 53

4.1.3 Plasmid profiles of *Lactobacillus* test strains 57

4.2 EFFECT OF PLASMID CURING ON BILE TOLERANCE OF *LACTOBACILLUS* SPP. 59

4.2.1 Plasmid curing and screening for loss of bile tolerance 59

4.2.2 Comparison of the plasmid profiles of strain ATCC 43121 and the cured strains 61

4.2.3 Comparison of the bile tolerance of *L. acidophilus* ATCC 43121 and its cured variants 69

4.2.4 Comparison of the fermentation profiles of *L. acidophilus* ATCC 43121 and the cured strains 74

4.3 INCREASED BILE TOLERANCE OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* 74

4.3.1 Selecting for increased bile tolerance 74

4.3.2 Comparison of the bile tolerance of strains J9, J9-S, and J9-V on bMRS agar 76

4.3.3 Comparison of the viability of strains J9, J9-S, and J9-V in bMRS broth 78

4.3.4 Comparison of *L. delbrueckii* subsp. *bulgaricus* strain J9 and variants J9-S and J9-V by fermentation profiles and coagulation of skim milk 78

5 DISCUSSION AND CONCLUSIONS 83

6 REFERENCES 92

LIST OF TABLES

Table		Page
1	Strains of *Lactobacillus* spp. used in the study	38
2	Initial screening of selected *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *Lactobacillus* GG strains for growth in bMRS broth and on bMRS agar after 72 h incubation at 35°C	52
3	Fermentation profiles of the experimental strains according to API 50 CHL test strips	54
4	Fermentation profiles of selected *Lactobacillus* strains on carbohydrate fermentation agar	55
5	Identification of experimental strains by API 50 CHL testing	56
6	Results of differential counting after curing of strains of *Lactobacillus* with novobiocin at 45°C	60
7	Counts of *L. acidophilus* ATCC 43121 and variants II-35, 6-9, 6-10, 7-13, and 26-1 on MRS and 2 and 5% bMRS agar after 48 h at 35°C	70

8 Maximum population of strains ATCC 43121 and 26-1 and hours of incubation at 35°C in various concentrations of bile....73

9 The fermentation profiles of *L. acidophilus* ATCC 43121 and variants II-35, 6-9, 6-10, 7-13, and 26-1....75

10 The colony counts of J9, J9-S, and J9-V on bMRS agar after 72 h incubation at 35°C....77

11 Maximum population of *L. delbrueckii* subsp. *bulgaricus* strains J9, J9-S and J9-V and time of incubation at 35°C at different concentrations of bile....79

12 Comparison of the fermentation profiles of strains J9, J9-S, and J9-V on carbohydrate fermentation agar....82

LIST OF FIGURES

Figure **Page**

1 Schematic presentation of the plasmid profiles of *Lactobacillus* spp. strains *L. acidophilus* ATCC 33198, *Lactobacillus* GG ATCC 53103, *L. acidophilus* BG2-FO4, and *L. acidophilus* ATCC 43121, with the plasmids of *Escherichia coli* V517 as the size marker.....58

2 Plasmid profiles of *L. acidophilus* ATCC 43121, and the cured variants 6-9, 6-10, 7-13, and 26-1, with plasmids of *Escherichia coli* V517 as a size marker.....63

3 Plasmid profiles of *L. acidophilus* ATCC 43121 and variant 6-10 after large scale plasmid isolation, with plasmids of *Escherichia coli* V517 as a size marker.....64

4 Migration of plasmids of *Escherichia coli* V517 relative to molecular size. Standard curve used for estimation of the size of the plasmids of *L. acidophilus* ATCC 43121.....65

5 Plasmid profiles of *L. acidophilus* ATCC 43121 and variant 6-10 after purification on cesium chloride - ethidium bromide density gradient, with plasmids of *Escherichia coli* V517 as a size marker.....66

6 Plasmid profiles of *L. acidophilus* ATCC 43121 and variant 26-1 after large scale plasmid isolation, with plasmids of *Escherichia coli* V517 as a size marker.....67

7 Plasmid profiles of *L acidophilus* ATCC 43121 and variant 26-1 after purification on cesium chloride - ethidium bromide density gradient, with plasmids of *Escherichia coli* V517 as a size marker.....68

8 Comparison of the viability of *L. acidophlius* ATCC 43121 and variant 26-1 in 0, 0.15, 0.3, 0.6, 1, 2, and 5% bMRS.....72

9 Comparison of viability of *L. delbrueckii* subsp. *bulgaricus* strains J9-S, and J9-V in 0, 0.15, 0.3, 0.6, and 1% bMRS.....80

10 Comparison of viability of *L. delbrueckii* subsp. *bulgaricus* strains J9, J9-S, and J9-V in 2, 2.5, 3, 4, and 5% bMRS.....81

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	adenosine triphosphate
B.	*Bifidobacterium*
bMRS	Lactobacilli MRS medium containing bile
C.	*Clostridium*
CBH	conjugated bile acid hydrolase
cbh	conjugated bile acid hydrolase gene
cfu	colony forming units
CH	chromosomal
DCA	sodium deoxycholate
DNA	deoxyribonucleic acid
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
GIT	gastrointestinal tract
L.	*Lactobacillus*
LAB	lactic acid bacteria
LBS	Lactobacillus Selective medium
log	logarithmic
LPS	lipopolysaccharide
MDa	megadalton
MIC	minimum inhibition concentration
$MIC_{45°C}$	minimum inhibition concentration at 45°C
MRS	Lactobacilli MRS medium
OC	open circular
R	rough

RNAase A	ribonuclease A
rRNA	ribosomal ribonucleic acid
S	smooth
SDS	sodium dodecyl sulphate
sp.	species (singular)
spp.	species (plural)
subsp.	subspecies
TBE	Tris-borate
TCA	sodium taurocholate
TCDCA	sodium taurochenodeoxycholate
TDCA	taurodeoxycholate
TE	Tris-EDTA
TUDCA	sodium tauroursodeoxycholate
UV	ultraviolet

1 INTRODUCTION

Fermented milks have been part of the human diet since the milking of animals was started (Kosikowski, 1977). The milk soured naturally and was preserved in that way. The souring of milk was due to the action of naturally occurring lactic acid bacteria (LAB) in the milk (Sharpe, 1979). First the use of the LAB was fortuitous but over the past 100 years the naturally occurring fermentation processes have become more controlled and have evolved into the modern-day dairy fermentation industry. According to Sharpe (1979), fermented dairy products account for approximately 20% of the total economic value of fermented foods worldwide.

Lactic acid bacteria are a large group of bacteria, that are Gram positive, nonsporeforming, carbohydrate fermenting, lactic acid producing rods and cocci, that are principally acid tolerant, catalase negative and facultatively anaerobic or microaerophilic; they are also usually nonmotile and do not reduce nitrate (Aguirre and Collins, 1993). Lactic acid bacteria comprise the genera: *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Enterococcus*, *Gemella*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Vagococcus* (Pot et al., 1994). Bifidobacteria are phylogenetically distant relatives of the LAB, despite of the fact that they were previously associated with the LAB and *Bifidobacterium bifidum* was previously classified as *Lactobacillus bifidus* (Aguirre and Collins, 1993). Traditionally, the LAB associated with foods belonged to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* were the LAB associated with foods. The development of molecular genetic techniques, e.g. 16S rRNA sequencing, DNA-rRNA hybridization studies, and the guanine + cytosine (G+C) content of the DNA, has lead to major reorganization of the taxonomy of the LAB, that was previously based on phenotypical characteristics.

The main focus of this review will be the *Lactobacillus* spp. of the LAB. Some species of the genus *Lactobacillus* are part of the indigenous microflora of humans, being found in the oral cavity, the gastrointestinal tract, and the urogenital tract (Hill and Marsh, 1990; Kandler and Weiss, 1986). Strains of *Lactobacillus* spp. have been used and researched for their value as probiotic microorganisms. The word "probiotic" is derived from a Greek words meaning 'for life' (Fuller, 1992). Fuller (1989) defined the term "probiotic" as: "A live microbial feed supplement which beneficially affects the host animal by improving its microbial balance". The definition of probiotics was broadened by Huis In't Veld and Havenaar (1991) to: "A mono- or mixed culture of live microorganisms, which applied to man or animal (e.g. as dried cells or as a fermented product), affects beneficially the host by improving the properties of the indigenous microflora." Among the *Lactobacillus* spp. currently used in probiotic preparations are *L. acidophilus*, *L. brevis*, *L. casei* subsp. *rhamnosus* (*Lactobacillus* GG), *L. cellobiosus*, *L. delbrueckii* subsp. *bulgaricus*, *L. fermentum*, *L. helveticus*, *L. lactis*, *L. plantarum*, and *L. reuteri* (Fuller, 1992; O'Sullivan et al., 1992).

In addition to lactobacilli, *Bifidobacterium* spp. is the other genus being widely used and researched for its value as a probiotic (O'Sullivan et al., 1992). The bifidobacteria used as probiotics include: *Bifidobacterium bifidum*, *B. longum*, *B. infantis*, *B. brevis*, and *B. adolescentis*. Other bacteria that have been used in probiotic preparations include: *Streptococcus thermophilus*, *Enterococcus faecium*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Streptococcus intermedius* (*Streptococcus anginosus*), *Leuconostoc mesenteroides* subsp. *dextranicum*, *Pediococcus acidilactici*, *Propionibacterium freudenreichii*, *Escherichia coli*, and *Bacillus* sp. (Fuller, 1992; O'Sullivan et al., 1992). Yeasts (*Saccharomyces*

cerevisiae, *Saccharomyces boulardii*, and *Candida pintolepsii*) and moulds (*Aspergillus niger* and *Aspergillus oryzae*) are mainly used in probiotic products for animals, but also for humans (Fuller, 1992; McFarland and Bernasconi, 1993).

The basis for using probiotics is the protective effect of the intestinal microflora against pathogens (Fuller, 1989, 1991). This is called either barrier effect, colonization resistance, or competitive exclusion. The lack of intestinal microflora makes germ-free animals more susceptible to disease than animals with normal intestinal microflora. Only 10 cells of *Salmonella enteritidis* were sufficient to kill a germ-free mouse whereas almost 10^9 viable organisms were needed to kill a mouse with normal intestinal microflora (Collins and Carter, 1978).

Under normal conditions, the human gastrointestinal tract is a stable ecosystem, but some dietary and environmental factors can cause disturbances in the balance of the normal flora (Fuller, 1989, 1991; Heimdahl and Nord, 1979; Orrhage et al., 1994; Zoppi et al., 1982). It is well established that antibiotic treatment can disrupt the normal microflora and allow the growth of opportunistic pathogens, such as *Clostridium difficile*, that cause diarrhea or pseudomembraneous colitis. Pseudomembraneous colitis caused by *C. difficile* has been cured by administration of fecal enemas from healthy adults (Bowden et al., 1981), and *Lactobacillus* GG strain has been shown to prevent relapse of pseudomembraneous colitis caused by *C. difficile* (Gorbach et al., 1987).

Probiotics may be useful in situations such as those described above where the normal microflora has been reduced, and also in cases where the normal microflora has not developed naturally because of a sterile environment, e.g. in neonatal farm animals

(Fuller, 1991). In addition to (re-)establishing the intestinal microflora, there are claims of various beneficial effects of probiotics, such as alleviation of lactose intolerance, anticholesteremic effect, anticarcinogenic activity, stimulation of the immune system, improved nutritional value of food, alleviation of constipation, prevention/reduction of diarrhea, and growth promotion of farm animals (Fuller, 1989; Goldin and Gorbach, 1992)

The gastrointestinal tract (GIT) is a complex ecosystem, which is difficult to study, and it is not known exactly how probiotics work in this environment. What happens under in vitro conditions might not be true in vivo. Some suggested mechanisms include production of antimicrobial substances (volatile fatty acids, lactic acid, bacteriocins), competition with pathogens for adhesion receptors, competition for nutrients and stimulation of host immunity (Fuller, 1991).

According to Havenaar et al. (1992) the beneficial effects of probiotics on the microbial balance of the host can be exerted at different locations in the body: a) in the mouth or gastrointestinal tract; b) in the upper respiratory tract; c) in the urogenital tract. In this study, the emphasis will be on the potential effect of orally taken probiotics for the gastrointestinal tract (GIT). For probiotic organisms to be effective they have to overcome the defense mechanisms of the body, which include the enzymes in the oral cavity (e.g. amylase, lysozyme); enzymes (pepsin, lipase) and extreme acidity of the stomach; concentration of bile, pancreatic juice and mucus in the small intestine; and the constant flushing by peristalsis (Havenaar et al., 1992). For prolonged survival in the GIT after consumption, the microorganisms must have a short generation time and/or the ability to colonize the intestinal surfaces by adhering to the epithelial cells.

Even if the probiotic organism survives the physical and chemical barriers in the GIT, it is likely to be in a stressed state which may lessen its competitive edge in relation to other microorganisms in the intestines. However, some authors suggest that probiotic microorganisms can have a beneficial effect even if partially or totally inactivated, and even if they do not colonize the intestine they can still have a transient beneficial effect (Fuller, 1991; Goulet and Matar, 1993; Havenaar et al., 1992). If the microorganisms only have a transient effect, they have to be taken continuously in a sufficient concentration to maintain them in the GIT. The use of so-called prebiotics has been suggested as an alternative to probiotic organisms (Anon., 1995). Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of colonic bacteria, thus improving the host's health. Prebiotics include for example some non-digestible oligosaccharides (oligofructose, inulin) that enhance the growth of bifidobacteria (Anon., 1995).

A potential probiotic strain might have several important properties required for survival and establishment in the GIT but be lacking some of the other important criteria required. In a situation like this, gene technology may aid in constructing an improved strain. This study concentrated on the effects of bile on lactobacilli. *L. delbrueckii* subsp. *bulgaricus* was reported to be sensitive to bile compared with *L. acidophilus* (Gilliland and Speck, 1977b). *L. delbrueckii* subsp. *bulgaricus* has a long tradition of use as a yogurt starter, but the evidence for its survival in and colonization of the GIT is lacking (Conway et al., 1987; Herter and Kendall, 1908; Mital and Garg, 1992; Rahe, 1915; Robins-Browne et al., 1981). *L. acidophilus* survives in the GIT and has been widely researched for its use as a probiotic organism. The differences in bile tolerance within *Lactobacillus* sp. suggested that the bile

tolerance of these strains could be plasmid-mediated. If bile tolerance is plasmid-mediated, a bile sensitive strain such as *L. delbrueckii* subsp. *bulgaricus* could be transformed with a plasmid for bile tolerance. Injury due to freezing, freeze drying and vacuum drying has been reported to increase the sensitivity of LAB to bile (Brennan et al., 1986; Johnson et al., 1984; Kole and Altosaar, 1984).

In this study, *Lactobacillus* spp. was used as a model to study the possibility of alteration of a strain's bile tolerance. The objectives of this study were:

(1) To find bile tolerant and bile sensitive strains of *Lactobacillus* spp.

(2) To determine if the bile tolerance of these strains of *Lactobacillus* could be altered,

(a) by curing the bile tolerant strains of their plasmids and testing the impact of loss of a plasmid on the bile tolerance of the strain, and

(b) by exposing a bile sensitive strain to bile to adapt the strain to bile.

2 REVIEW OF THE LITERATURE

2.1 HISTORICAL REVIEW OF PROBIOTICS (with emphasis on the lactobacilli)

As early as 76 BC, the Roman historian Plinio advocated the use of fermented milks for the treatment of various (pathological forms of) gastrointestinal infections (Bottazzi, 1983). The first scientific research on probiotics was conducted by Elie Metchnikoff and Henry Tissier at the turn of the 20th century (Bibel, 1988; Hughes and Hoover, 1991). They created an awareness of the effect of intestinal microflora on the health of the host, and the possibilities of improving the indigenous flora by consuming fermented milk products or live microorganisms.

Elie Metchnikoff (1845-1916) was initially concerned with the adverse effects (autointoxication, opportunistic infections) of intestinal microflora on a host, to the extent of suggesting surgical removal of the colon for the prolongation of life, a practice which he soon abandoned (Bibel, 1988). By studying germ-free animals, Metchnikoff demonstrated that microorganisms were not required for normal digestion. During the cholera pandemic at the end of the 19th century, Metchnikoff studied the infectivity of cholera vibrio and found that simultaneous inoculation of "favorable bacilli" and cholera vibrios into the gastrointestinal tract of laboratory animals did not result in infection. The results of the cholera experiments made Metchnikoff realize the protective effect of healthy intestinal flora against pathogens.

After the observation that long-living Bulgarian peasants consumed large quantities of soured milk in their diet, Metchnikoff conducted his studies with sour milk (Bibel,

1988). He suggested that the intestinal microflora could be controlled by transforming the "wild population of the intestine into a cultured population", which could be interpreted as replacing undesirable microorganisms with beneficial microorganisms. The 'favorable bacilli' produced lactic acid and inhibited the growth of the putrefactive microorganisms in vitro. In addition, lactic acid helped in the treatment of diarrhea. Metchnikoff and his colleagues found that a strain from a Bulgarian culture survived in the intestine and produced a high level of lactic acid.

Although Metchnikoff was cautious not to make unwarranted claims about the benefits of consuming fermented milks in his publications, and felt the need for extensive research in this area, the public interest in lactobacilli and fermented milks was tremendous. The Pasteur Institute started selling "Bulgarian bacillus" cultures (Bibel, 1988; Fuller, 1992). Metchnikoff allowed his name to be used on a fermented milk product. This was the start of the industrial production of yogurt and other fermented milks. Despite his hypothetical approach, Metchnikoff was interpreted by the public, and especially by Americans, to have claimed that "curdled milk is able to prolong life", which hurt his scientific reputation. After Metchnikoff's death in 1916 the interest in bacteriotherapy declined but the research continued in the field that is now known as probiotics. By now, several claims on the health benefits of consumption of live lactobacilli in fermented milk products and other products have been made and several other microorganisms have been added to the list of potential probiotic candidates. There are still many questions to be answered.

In 1921, Rettger and Cheplin, in studies at Yale University, which became the centre of probiotic studies after Metchnikoff's death, reported that the "Bulgarian bacillus" was destroyed in the human intestine (Bibel, 1988; Fuller, 1992). Instead, they

promoted the use of intestinal isolates of *Lactobacillus acidophilus* (Bibel, 1988; Fuller, 1992). Besides its survival in the GIT, the interest in *L. acidophilus* was based on the belief of it being the dominant *Lactobacillus* in the intestine. According to a personal communication by D.J. Bibel to Fuller (1992), "It is impossible to know with any certainty which species Metchnikoff and his contemporaries were studying but it is likely that unintentional mixtures of lactobacilli were sometimes used." According to Fuller (1992), the initial work done by Metchnikoff and his colleagues was likely done with an organism closely related to *L. delbrueckii* subsp. *bulgaricus*, but it is possible that a *Lactobacillus* strain that was not *L. delbrueckii* subsp. *bulgaricus* was the one that survived in the intestinal study done by Metchnikoff. The ability of *L. delbrueckii* subsp. *bulgaricus* to survive in and colonize the intestine has been questioned in other studies (Conway et al., 1987; Herter and Kendall, 1908; Mital and Garg, 1992; Rahe, 1915; Robins-Browne et al., 1981), and *L. bulgaricus* is not normally isolated from the human intestine (Molin et al., 1993; Robins-Browne et al., 1981). *L. acidophilus*, *L. brevis*, *L. casei*, *L. cellobiosus*, *L. catenaforme*, *L. crispatus*, *L. fermentum*, *L. helveticus*, *L. lactis*, *L. leichmanii*, *L. minutus*, *L. plantarum*, *L. salivarius* subsp. *salivarius*, and *L. rogosae* are the *Lactobacillus* spp. most commonly isolated in human feces (Finegold et al., 1983).

Lactobacillus spp. are among the dominant genera in the jejunum (Johansson et al., 1993). However, the bacterial concentrations in the jejunal mucosa are relatively low, and some of the bacteria may be temporary contaminants from the upper respiratory tract. According to Mitsuoka (1978), the approximate log number of lactobacilli per gram of GIT contents are as follows: oral cavity 7, stomach 4, duodenum 2, jejunum 3, ileum 6, cecum 7, and rectum 6. About 9 to 12 log lactobacilli per gram of dry feces were isolated in human feces (Finegold et al., 1977).

For historical reasons, *L. delbrueckii* subsp. *bulgaricus* strains are still included in some probiotic preparations (Fuller, 1992), and yogurt and other fermented milk products can be used as carriers for probiotic bacteria (Hose and Sozzi, 1991). The research on probiotics has concentrated on other *Lactobacillus* strains, especially *L. acidophilus*, and other bacteria, even yeasts (Fuller, 1992; O'Sullivan et al., 1992). *Lactobacillus* GG is a more recent, promising human isolate that has been widely researched for its clinical applications (Goldin et al., 1992; Isolauri et al., 1991; Kaila et al., 1992; Ling et al., 1994; Meurman et al., 1994; Oksanen et al., 1990; Saxelin et al., 1991; Silva et al., 1987). In addition to their traditional use as yogurt starters, *Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus* have been added to certain dairy products to enhance flavour, because dairy products containing only *L. acidophilus* and bifidobacteria are not found to be very palatable by consumers. There are other challenges in using *L. acidophilus* and bifidobacteria as dairy starters (Daly, 1991; Marshall, 1991; Mital and Garg, 1992). *L. acidophilus* grows slowly in milk and requires growth promoting supplements (such as yeast extract, casein peptone, tomato juice) for enhanced growth (Mital and Garg, 1992). In mixed cultures, *L. acidophilus* is reduced to a minor portion of the final population because of its slower growth compared with other cultures. This has lead to the development of products which incorporate milk, yogurt or ice cream as carriers for the delivery of large numbers of *L. acidophilus*. Many investigators have demonstrated that *L. acidophilus* grows well in soy milk, in which the major fermentable sugar is sucrose instead of lactose. The resulting product is described as an acceptable, yoghurt-like product (Mital and Garg, 1992).

The acidity of fermented dairy products can cause loss of viability of the microorganisms during prolonged storage (Mital and Garg, 1992). The acidity should

be 0.6 to 0.7% lactic acid or lower for a sufficient *L. acidophilus* population to survive for 2 to 3 weeks. According to the review on acidophilus milk products by Mital and Garg (1992), a daily intake of 1 to 2 x 10^9 *L. acidophilus* has been recommended for replenishing lactobacilli in the intestines. According to Marshall (1991), there are approximately 10^8 live microorganisms per ml of fermented milks. Dried and freeze-dried preparations contain cells for which growth is arrested and resuscitation is necessary (Marshall, 1991). On reconstitution of these bacteria, less than 1% may be viable. Cell injury due to drying and freeze-drying can reduce the bile tolerance of a microorganism (Brennan et al., 1986, Johnson et al., 1984).

It is possible to take probiotic bacteria as a pill or dietary supplement. Robins-Browne et al. (1981) reported that *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*, in a commercial lyophilized preparation (Lactinex), survived the acidity of the stomach when taken with milk, but *L. acidophilus* survived better in the jejunum and was recovered more often and in slightly greater numbers than *L. delbueckii* subsp. *bulgaricus* in samples obtained from the jejunum. However, Robins-Browne et al. (1981) found no indication of long-term colonization of the proximal small intestine by these lactobacilli. *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* only persisted in elevated numbers for about 3 hours in fasting individuals and for up to 6 hours in nonfasting individuals. Johansson et al. (1993) reported increased levels of lactobacilli (*L. plantarum*, *L. casei* subsp. *rhamnosus*, *L. reuteri*, *L. agilis*) on the jejunal mucosa after oral administration of 100 ml of fermented oatmeal soup containing 5 x 10^6 cfu/ml of each of the 19 *Lactobacillus* test strains (*L. salivarius*, *L. plantarum*, *L. casei* subsp. *pseudoplantarum*, *L. jensenii-L. gasseri*, *L. acidophilus*, *L. crispatus*, *L. casei* subsp. *rhamnosus*, *L. reuteri*, *L. agilis*, and other *Lactobacillus* spp.). The test strains were detected 11 days after the end of administration, which indicated colonization. Johansson et al. (1993) did not isolate the *L. acidophilus*-like

test strains from the mucosa of any of the 13 subjects, but reported the dominance of two *L. plantarum* strains. Lidbeck et al. (1987) observed a significant increase in the number of lactobacilli in the anaerobic fecal microflora of 9 out of 10 subjects within 7 days of oral administration of 250 ml of fermented milk product containing 5×10^8 to 2×10^9 cfu of *L. acidophilus* per ml. Conway et al. (1987) reported that *L. acidophilus* (two strains tested) survived better in human gastric juice than *L. delbrueckii* subsp. *bulgaricus* (one strain tested), which in turn survived better than *S. thermophilus*. The addition of skim milk increased the survival of all strains (Conway et al., 1987). *L. delbrueckii* subsp. *bulgaricus* survived poorly in human gastric juice when tested in vivo in two subjects, but addition of skim milk extended the survival of *L. delbrueckii* subsp. *bulgaricus* to 40 minutes (Conway et al., 1987).

2.2 TAXONOMY OF THE *LACTOBACILLUS* SPP.

Orla-Jensen (1919) divided *Lactobacillus* sp. into three subgenera on the basis of their optimum growth temperatures and end-products of fermentation: *Thermobacterium, Streptobacterium,* and *Betabacterium.* To accomodate several newly described species, Kandler and Weiss (1986) omitted growth temperature and cell morphology as essential characteristics for discrimination the *Lactobacillus* spp., and divided the genus *Lactobacillus* into three physiological groups: obligately homofermentative, facultatively heterofermentative, and obligately heterofermentative groups which corresponded to the division by Orla-Jensen, but also included the newly described species (Pot et al., 1994). The classification of *Lactobacillus* spp. by the new techniques does not correlate with the traditional methods of classification (Collins et al., 1991). Comparative analysis of 16S rRNA sequence data of 53 species of *Lactobacillus* and 24 reference strains of related genera (*Aerococcus*,

Carnobacterium, *Enterococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Tetragenococcus*) resulted in three phylogenetically distinct clusters (Collins et al., 1991). Cluster 1 (the *L. delbrueckii* group), with *L. delbrueckii* as the type species of the genus, consisted solely of obligately homofermentative species. *L. delbrueckii* subsp. *bulgaricus* was not included in the study by Collins et al. (1991), but *L. delbrueckii* subsp. *delbrueckii* (NCD0213) and *L. delbrueckii* subsp. *lactis* (NCD01438) were among the strains studied, and were included in group 1, as well as *L. acidophilus* (NCD01748) and *L. acidophilus* (group A3) (NCD02745). Cluster 2 (the *L. casei* group) included 32 *Lactobacillus* species as well as 5 *Pediococcus* species. The members of cluster 2 were mostly heterofermentative, but 5 obligately homofermentative species were also included in the group. Cluster 3 (the *Leuconostoc paramesenteroides* group) contained the atypical heterofermentative lactobacilli (*L. confusus*, *L. kandleri*, *L. minor*, *L. viridescens*) and *Leuconostoc paramesenteroides*, to which the heterofermentative lactobacilli are closely related (Collins et al., 1991). Collins et al. (1993) proposed that cluster 3 should be reclassified as a new genus *Weissella*, and should include a new species *Weissella hellenica*, that was isolated from Greek fermented sausage.

2.2.1 *L. delbrueckii* subsp. *bulgaricus*

The strain used by Metchnikoff was called the "Bulgarian bacillus", which is most probably the same strain as *Bacillus bulgaricus*, that was classified by Orla-Jensen as *Thermobacterium bulgaricum* (Orla-Jensen, 1919). *Thermobacterium bulgaricum* was later named *Lactobacillus bulgaricus*, and is now called *Lactobacillus delbrueckii* subsp. *bulgaricus* (Fuller, 1992). Today *L. delbrueckii* subsp. *bulgaricus* is used as

a starter culture together with *Streptococcus thermophilus* in manufacture of yogurt and other fermented dairy products.

2.2.2 *L. acidophilus*

L. acidophilus was first isolated from infant feces in 1900 and was called *Bacillus acidophilus* (Johnson et al., 1980). The description of the species remained vague, and eventually the heterogeneity of the species was recognized. The physiological characteristics were not adequate to define *L. acidophilus* as the species that is now referred to as the *L. acidophilus* group, because it evolved into six separate species. Johnson et al. (1980) divided the *L. acidophilus* strains into six distinct DNA homology groups, designated as A1, A2, A3, A4, B1, and B2. Subgroup A1 retained the name *L. acidophilus*. Subgroup A2 was found to be homologous with the existing strain *L. crispatus* (Cato et al., 1983). Lauer and Kandler (1980) proposed subgroup B1 as a new species, *L. gasseri*. Fujisawa et al. (1992) described two new species within the *L. acidophilus* DNA homology groups, namely subgroup A4 as *L. gallinarum* and subgroup B2 as *L. johnsonii*. Subgroup A3 was recognized as being the same as the existing species *L. amylovorus*.

Johnson et al. (1987) suggested that the genetic heterogeneity of *L. acidophilus* strains could be a reason for controversy on the health benefits of *L. acidophilus* in the human GIT. According to Johnson et al. (1987), strains that either are not *L. acidophilus* or that do not have the assumed traits of *L. acidophilus* may have been used in some studies.

2.2.3 *L. casei*

The *L. casei* subgroup has also gone through a major nomenclatural reorganization (Pot et al., 1994). Collins et al. (1989), among others, observed little DNA homology between the designated type strain of *L. casei* subsp. *casei*, ATCC 393 (NCDO 161), and the strains belonging to the *L. casei* subsp. *casei*, *alactosus*, *pseudoplantarum*, and *tolerans*. Based on these results, Collins et al. (1989) reclassified *L. casei* subsp. *alactosus*, *L. casei* subsp. *pseudoplantarum*, *L. casei* subsp. *tolerans*, and the majority of *L. casei* subsp. *casei* strains as a new species, *L. paracasei*. However, based on the results from total soluble cell protein profiles and DNA:DNA hybridizations, Dellaglio et al. (1991) requested the designation of strain ATCC 334 in place of ATCC 393 as the type strain of *L. casei* subsp. *casei*, as well as the rejection of the species name *L. paracasei*, but the request was denied (Wayne, 1994). Dellaglio et al. (1991) confirmed the reclassification of *L. casei* subsp. *rhamnosus* as species *L. rhamnosus*, as proposed by Collins et al. (1989).

2.2.4 *Lactobacillus* GG

Lactobacillus GG is a widely researched human fecal isolate which has been screened for growth in 0.15% bile (oxgall), grows at pH 3.0, exhibits in vitro adherence to intestinal mucosal cells, and produces an antimicrobial substance (Goldin et al., 1992; Silva et al., 1987). *Lactobacillus* GG was isolated from the feces of a healthy person (Silve et al., 1987). Goldin et al. (1992) reported that the electrophoretic pattern of soluble proteins of *Lactobacillus* GG most closely resembled that of *L. casei* subsp. *rhamnosus*. The fermentation profile of *Lactobacillus* GG differed from *L. casei* subsp. *rhamnosus* in that *Lactobacillus* GG did not ferment lactose, maltose or

sucrose. According to Goldin et al. (1992), the colonial morphology of *Lactobacillus* GG is distinct and facilitates its recognition in a mixed culture.

2.3 BILE IN THE HUMAN BODY

To better understand the conditions that probiotic bacteria encounter in the intestines, a brief overview of bile metabolism in humans is presented. Results from studies on the physiological bile concentration in the intestines will assist in determining the extent of bile tolerance required for probiotic strains.

2.3.1 Bile metabolism in humans

Bile is synthesized in the liver, and stored and concentrated in the gallbladder (Sturkie, 1981). Soon after ingestion of food the gallbladder starts to empty as a consequence of hormonal and nervous mechanisms, and bile is released into the duodenum through the bile duct (Borgström et al., 1985; Sturkie, 1981). In the absence of a gallbladder, bile is secreted constantly, at a slow rate, directly into the intestine, because the liver secretes bile continuously.

The water content of human hepatic bile and of human gallbladder bile is 97 to 98%, and 82%, respectively. Besides water, human bile contains bile salts (0.7%), bile pigments (0.2%), cholesterol (0.06%), and phospholipids (mainly lecithin; 0.02%) (Sturkie, 1981). The pH of human hepatic and gallbladder bile is 7.4 and 5 to 6, respectively (Sturkie, 1981).

The primary bile acids, cholic acid (3α–, 7α–, 12α-trihydroxy-5β-cholanoic acid) and chenodeoxycholic acid (3α–, 7α-dihydroxy-5β-cholanoic acid) are synthesized from cholesterol by the liver (Hylemon and Glass, 1983; Spallholz, 1989). Cholic acid is the main bile acid. Human bile also contains a secondary bile acid, deoxycholic acid (3α–, 12α-dihydroxy-5β-cholanoic acid) (Drasar and Hill, 1974; Hylemon and Glass, 1983). Secondary bile acids are formed by 7-dehydroxylation of the primary bile acids. Deoxycholic acid is derived from cholic acid and lithocholic acid (3α-hydroxy-5β-cholanoic acid) is derived from chenodeoxycholic acid. The carboxyl group of bile acids can be conjugated to either glycine or taurine (ethylamine sulfonic acid) to form conjugated bile acids (Hylemon and Glass, 1983). Normally the glycine conjugates predominate in humans (Dowling, 1972). Over 95% of the bile acids in hepatic and gallbladder bile are conjugated (Sung et al., 1993). Approximately one-quarter to one-third of the total bile acids are secondary bile acids, deoxycholic acid and occasionally trace amounts of lithocholic acid (Dowling, 1972).

In the small intestine, the water soluble bile salts (Na, K) emulsify dietary lipids to facilitate their hydrolysis by lipases and their absorption by the intestine (Spallholtz, 1989; Sung et al., 1993). Because bile salts contain both polar and nonpolar regions they are effective detergents (Stryer, 1988). Bile salts form mixed micelles with phospholipids (predominantly lecithin) and cholesterol in the bile, and with fatty acids, monoglycerides, and fat soluble vitamins in the intestine (Sung et al., 1993). The majority of bile salts (ca. 90%) are reabsorbed from the terminal ileum by an active transport mechanism, and returned to the liver by the enterohepatic circulation (Hylemon and Glass, 1983). Free bile acids are absorbed by passive diffusion from the small or large intestine. A portion of the bile salts (ca. 2 to 10%) escapes to the

large intestine and about 200 to 600 mg of bile salts are excreted daily in the feces (Hylemon and Glass, 1983; Spallholz, 1989).

In feces the bile acids are entirely deconjugated, and the majority of these free bile acids are the secondary bile acids deoxycholic acid and lithocholic acid (Dowling, 1972; Hylemon and Glass, 1983). Among other microbial transformations of bile acids, intestinal microflora is responsible for the hydrolysis (deconjugation) of bile acid conjugates (see Section 2.4), which is the first step in bile degradation, as well as for bile acid dehydroxylation, yielding secondary bile acids (Dowling, 1972; Hill and Drasar, 1968; Hylemon and Glass, 1983). At least 15 to 20 different bile acids are known to result from the transformation of primary bile acids by human intestinal microflora (Dowling, 1972; Hylemon and Glass, 1983).

2.3.2 Physiological concentration of bile acids

The highest concentration of bile acids in the duodenum occurs generally between 15 and 30 minutes after ingestion of a meal, when the gallbladder empties into the duodenum (Sjövall, 1959). The emptying of the gallbladder increases the concentration of bile acids in the proximal small intestine, but it does not affect the concentration of bile acids in the distal part of the small intestine. Sjövall (1959) investigated the concentration of bile acids in the intestinal contents of seven normal individuals and one individual without a gallbladder, during the absorption of a liquid test meal, and found that the highest concentration of bile acids in each individual varied between 13 and 46 mmol/l of intestinal contents. The minimum concentration of bile acids occurred 45 minutes to 2 hours after the test meal, and was between 2.5 and 4.0 mmol/l of intestinal contents. All of the bile acids were conjugated, including the two samples from the distal small intestine. In the ileum, Sjövall (1959) recorded

a concentration of bile acids between 7.3 and 10.9 mmol/l of intestinal contents, 1 to 4 hours after the test meal. Most of the time, during the absorption of nutrients in the intestines, the concentration of bile acids throughout the intestines was between 2.5 and 10 mmol/l of intestinal contents. This was also the case in the subject without a gallbladder. According to Sjövall (1959), the proportions of different bile acids varied markedly between individuals.

Similar intestinal bile acid concentrations were reported by Mallory et al. (1973), who found that the concentration of total bile acids in the duodenum of 12 healthy individuals varied between 7.0 and 30.9 mmol/l. The mean was 20.0 mmol/l. According to Mallory et al. (1973), the removal of the gallbladder of three patients did not affect bile acid concentrations in the duodenum compared with other patients who had intact gallbladders. The concentration of unconjugated bile acids varied between 0 and 1.6 mmol/l in normal subjects. Somewhat contradictory to Sjövall's findings, Mallory et al. (1973) reported that the mean percentages of the four major bile acids varied only slightly from area to area in the intestines of normal subjects. Mallory et al. (1973) reported the following percentages of major bile acids in the intestines: 44 to 52% cholic acid, 27 to 34% chenodeoxycholic acid, 17 to 20% deoxycholic acid, and 1 to 3% lithocholic acid (Mallory et al., 1973).

According to Borgström et al. (1985), the bile salt content of intestinal content is approximately 5 to 10 mmol/l. Floch et al. (1972) reported bile concentrations of 6 to 10 mmol/l in the duodenum after a meal and 2 to 3 mmol/l during the resting state. These concentrations reported by Floch et al. (1972) were mainly conjugated bile acids. Floch et al. (1972) suggested that the rapid transfer of intestinal contents and the associated dilution factors may affect the actual concentration of bile in the

intestinal lumen. According to Floch et al. (1972), the concentration of bile acids in the feces was approximately 5 mmol/l.

2.4 DECONJUGATION AND DEHYDROXYLATION OF BILE ACIDS BY BACTERIA

2.4.1 Deconjugation of bile by intestinal bacteria

Several intestinal bacteria hydrolyze conjugated bile acids but the presence and extent of this activity varies considerably between microbial species (Hylemon and Glass, 1983). Generally, intestinal bacteria hydrolyze both glycine and taurine conjugates of bile acids. The enzyme responsible for bile deconjugation is conjugated bile acid hydrolase (CBH), that can also be named more specifically according to its specificity e.g. cholyltaurine hydrolase, cholylglycine hydrolase, etc. (Christiaens et al., 1992; Feighner and Dashkevicz, 1987). CBH has been found in the genera *Bacteroides*, *Bifidobacterium*, *Fusobacterium*, *Clostridium*, *Lactobacillus*, *Peptostreptococcus*, *Streptococcus*, and *Veillonella* (Hill and Drasar, 1968; Hylemon and Glass, 1983). A number of *Lactobacillus* species, both homolactic and heterolactic fermenters, have been reported to hydrolyze either glycine- or taurine-conjugated (or both types of) bile acids, including: *L. acidophilus*, *L. arabinosus*, *L. buchneri*, *L. plantarum*, *L. salivarius*, *L. brevis*, *L. casei*, *L. fermentum* (Dashkevicz and Feighner, 1987; Gilliland and Speck, 1977a; Shimada et al., 1969). All six of the fecal *L. acidophilus* isolates tested by Gilliland and Speck (1977a) deconjugated taurocholate, whereas only one of them deconjugated glycocholate. Gilliland and Speck (1977a) observed a requirement for a low redox potential for deconjugation activity of *L. acidophilus*.

Hill and Drasar (1968) found a relationship between the proportion of strains of *Bacteroides*, *Veillonella*, and *Bifidobacterium* strains able to deconjugate bile acids, and the source of the strains in the gastrointestinal tract. Only 5% of the oral *Bacteroides* strains deconjugated bile acids compared with 75% of the fecal strains. Many oral *Bacteroides* and *Bifidobacterium* strains were sensitive to bile. When Hill and Drasar (1968) subcultured some oral bacteria several times on a growth medium containing 10% Difco ox-bile (a treatment which resulted in only one of the bifidobacteria surviving), many of the strains gained the ability to deconjugate bile (5/5 bacteroides, 1/1 bifidobacteria and 5/12 veillonella). However, none of the *E. coli* strains and the yeasts isolated from feces deconjugated bile despite their previous exposure to bile. Repeated subculture of strains of *E. coli* and *Pseudomonas aeruginosa*, in the presence of bile, did not produce any bile deconjugating strains. *Pseudomonas* spp. were reported to be capable of utilizing bile acids as a sole carbon source (Leppik, 1989), but whether these strains deconjugated bile was not reported. Research by Leppik (1989) suggested that the ability to catabolize bile acids was chromosomally mediated. *Staphylococcus aureus* deconjugates bile, but is not an intestinal organism, so an explanation for its deconjugating ability is difficult to find.

The physiological significance of CBH to the lactobacilli has not been elucidated (Floch et al., 1972; Gilliland and Speck, 1977a; Tannock et al., 1989). Free bile acids are more inhibitory to bacteria (anaerobes and Gram-positive aerobes) than conjugated bile acids. Considering this, deconjugation and the resulting free bile acids may have a role in controlling bacterial populations in the intestine (Floch et al., 1972; Gilliland and Speck, 1977a). In an experiment by Floch et al. (1972), *Clostridium perfringens*, *Bacteroides fragilis*, *Lactobacillus* spp. or *Enterococcus* spp. were not inhibited

unless free bile acids were present. Deoxycholic and chenodeoxycholic acids were inhibitory at 1 and 2 mM concentrations, which are physiological concentrations in humans. Chenodeoxycholic acid has especially high inhibitory activity according to Floch et al. (1972). Certain bacterial strains are inhibited by the free bile acids that are produced as a result of their own deconjugating activity, which could mean that deconjugation of bile acids is an autoregulatory system in which individual bacteria control their own population (Floch et al., 1972). Deconjugation is very unlikely to serve as a detoxification mechanism because of the far greater inhibitory activity of free bile acids compared with conjugated bile acids (Tannock et al., 1989). Lactobacilli do not utilize the steroid moiety of the bile salt as a cellular precursor, but they might utilize the amino acid portion of the hydrolyzed bile salt (Tannock et al., 1989). In a study by Van Eldere et al. (1988), a strain of *Lactobacillus* from rat intestine was not stimulated by taurine.

Christiaens et al. (1992) isolated a conjugated bile acid hydrolase (CBH) gene (*cbh*) from *L. plantarum* and described its nucleotide sequence. By cloning the *cbh* gene into an *E. coli -Lactobacillus* shuttle vector and reintroducing the gene into the parental *Lactobacillus* strain, Christiaens et al. (1992) were able to achieve overproduction of the CBH enzyme. The deduced amino acid sequence was shown to have 52% similarity with a penicillin V amidase from *Bacillus sphericus*, but the physiological significance of these enzymes for bacteria has not been elucidated. Leer et al. (1993) obtained transformants deficient in CBH activity by disrupting the *cbh* gene of *L. plantarum*.

Hybridization experiments by Christiaens et al. (1992) indicated that the CBH enzyme is not plasmid encoded. Southern hybridization experiments indicated that the *cbh*

gene of *L. plantarum* was similar to the genomic DNA of intestinal CBH-active *Lactobacillus* spp. and that the *cbh* gene was well conserved among the different *Lactobacillus* spp. (*L. acidophilus*, *L. brevis*, *L. crispatus*, *L. fermentum*, *L. gasseri*, *L. plantarum*) tested. There is an indication that the deconjugating system is constitutive in *L. acidophilus* (Gilliland and Speck, 1977a). They observed that the deconjugating activity of resting cells of *L. acidophilus*, harvested from broth containing no taurocholate, was similar to the activity of cells that had been grown in broth containing taurocholate.

2.4.2 Effect of bile deconjugation on the health of the host

There is an indication of both a positive and a negative impact of the deconjugation of bile acids by the intestinal bacteria on the host. On the positive side, bile acids, especially in deconjugated and secondary form, may function to protect the host against intestinal pathogens (Floch et al., 1972). Gilliland et al. (1985) suggested that *L. acidophilus* assimilates cholesterol, and thereby has a hypocholesteremic effect in the host. Research by Klaver and Van Der Meer (1993) indicated that cholesterol was not assimilated but precipitated. Klaver and Van Der Meer (1993) found that the solubility of cholesterol was affected by the deconjugation of bile salts by *L. acidophilus* and *Bifidobacterium bifidum* when either of these strains was grown in the presence of cholesterol and oxgall under acidifying conditions. Deconjugated bile salts were less soluble at pH values lower than 6.0, and the precipitation of deconjugated bile salts caused co-precipitation of cholesterol. Cholesterol was fully recovered from supernatants and pellets, which indicated that cholesterol not metabolized. Klaver and Van Der Meer (1993) only observed significant precipitation of bile salts with deconjugating activity. The significance of these findings to the

hypocholesteremic effects observed in vivo is not yet fully elucidated. The physiological pH in the intestinal tract of humans is usually neutral to alkaline, but besides low pH, the presence of Ca^{2+} precipitates free bile acids (De Smet et al., 1994; Klaver and Van Der Meer, 1993). Deconjugated bile salts are not reabsorbed as efficiently by the intestine as conjugated bile salts (De Smet et al., 1994). In addition, deconjugated bile salts are adsorbed to a higher extent to bacterial cells and dietary fiber, which increases their fecal excretion. The bile acids excreted in the feces are removed from the total bile acid pool and will thus lead to de novo synthesis of bile acids from cholesterol. This is also thought to reduce the serum cholesterol levels.

Several results indicate that the deconjugation of bile acids has a negative impact on the host. Bacterial overgrowth in the small intestine can result in various clinical conditions described as the contaminated small bowel syndrome (Gracey, 1983). Free bile acids produced by bile salt-hydrolyzing bacteria are involved for example in steatorrhea and other diarrhea, carbohydrate malabsorption, and impaired uptake of protein. Steatorrhea occurs when bacterial overgrowth results in extensive bacterial deconjugation and, as a result, the concentration of conjugated bile acids in the small intestine falls below that normally required. This impairs lipid absorption and causes steatorrhea, a foul-smelling, fatty type of diarrhea (Gracey, 1983).

The detergent properties of bile acids may have detrimental effects in the colon (Van der Meer et al., 1991). Free secondary bile acids may promote colon cancer by damaging the colonic epithelial cells and thereby increasing proliferation of crypt cells. Deconjugated bile salts are involved in gallstone formation (Akiyoshi and Nakayama, 1990).

In production animals the bile deconjugating bacteria may play a role in the so-called growth depressing effect (Feighner and Dashkevicz, 1987, 1988; Tannock et al., 1989). Feighner and Dashkevicz (1987) showed that subtherapeutic levels of antibiotics decreased cholyltaurine hydrolase activity in ileal homogenates of chicks.

At the same time, weight gain and feed conversion rates of chicks were enhanced compared with the untreated control chicks. Germ-free chickens have been observed to grow faster and more efficiently than chickens with normal intestinal flora, with or without the feeding of subtherapeutic levels of antibiotics. These observations point to the growth depressing effect of the bile deconjugating intestinal microflora on the animal host.

2.5 BILE TOLERANCE OF BACTERIA

Bile tolerance is among the traits required for a probiotic microorganism, because concentrations of bile acids in the small intestine and the colon can inhibit or kill many bacteria (Goldin and Gorbach, 1992). Generally, bile acids are considered to influence the intestinal microflora (Floch et al., 1972). According to Havenaar et al. (1992), it is difficult to establish an exact level of bile tolerance for probiotic strains. Goldin and Gorbach (1992) stated that for probiotic bacteria to be effective they should be able to grow in agar medium containing 0.15 to 0.3% commercial oxgall.

2.5.1 Antibacterial properties of bile acids

The mechanism behind the antibacterial effect of bile salts has not been elucidated (Sung et al., 1993). The hydrophobicity of a bile salt is an important factor in

determining its antimicrobial activity (Sung et al., 1993). The more hydrophobic bile acids generally have fewer hydroxyl groups. Free bile acids are more hydrophobic than conjugated bile acids. The nonionized bile acids are more hydrophobic than the ionized bile salts. The more hydrophobic a bile salt is, the better its detergent effect, and the stronger its antimicrobial effect. The detergent effect possibly accounts for the antimicrobial effect of hydrophobic bile salts because of the greater solubilizing capacity for phospholipids and cholesterol. This theory was supported by the results of Sung et al. (1993), who reported that hydrophobic bile salts sodium taurodeoxycholate (TDCA) and sodium deoxycholate (DCA) at 100 mmol/l concentration were more inhibitory to *E. coli* than hydrophilic bile salts sodium taurocholate (TCA), sodium taurochenodeoxycholate (TCDCA), and sodium tauroursodeoxycholate (TUDCA) at the same concentration.

Sung et al. (1993) reported that the presence of lecithin at the same concentration as that present in gallbladder bile markedly reduced the antibacterial effect of TDCA, which suggests that the inhibitory effect of bile salts in vivo, especially in gallbladder bile, might be weaker than observed in experiments done in vitro. The detergent activity of bile salts may be diminished by the formation of mixed micelles with phospholipids, because the hydrophobic portion is involved in the aggregate. The results reported by Sung et al. (1993) suggested that the results achieved with bile salts and bile are not directly comparable because of the effect of lecithin in bile.

2.5.2 Effects of exposure of bacteria to bile

The cell structures responsible for bile resistance of *L. acidophilus* have not been elucidated but more is known about Gram-negative bacteria (Johnson et al., 1984).

The lipopolysaccharide (LPS) molecules on the cell surface of Gram-negative bacteria form a barrier against several molecules, including bile salts. Gram-negative bacteria have been reported to become bile sensitive after freezing or treatment with EDTA, procedures which cause damage to cell wall components such as LPS. Freeze-damage to the cell membrane has been reported in Gram-negative as well as in Gram-positive bacteria (Johnson et al., 1984). Recent studies have shown that the outer membrane of Gram-negative bacteria only slow down the influx of hydrophobic inhibitors, such as bile salts, to the cell, and Gram-negative bacteria require an energy-dependent drug efflux pump as protection against hydrophobic compounds (Ma et al., 1995).

L. acidophilus was reported to develop sensitivity to bile after freezing and thawing, after freeze drying, after vacuum drying, and after treatment with lysozyme or autolysin (Brennan et al., 1986; Johnson et al., 1984). Kole and Altosaar (1984) used bile resistance as a screening method to obtain strains of *Leuconostoc oenos* that are more resistant to lyophilization damage (Kole and Altosaar, 1984). The bile resistance that was lost was regained in the lysozyme and autolysin treated *L. acidophilus* cells after incubation in MRS broth, but not in the presence of penicillin, suggesting that peptidoglycan synthesis was needed for recovery. *L. acidophilus* cells surviving freezing and thawing developed sensitivity to 0.15% bile salts in Lactobacillus Selective (LBS) or Lactobacilli MRS (MRS) agars, but completely regained resistance after incubation in MRS broth. This was probably due to repair of freeze-injury. Johnson et al. (1984) studied some possible repair mechanisms to determine the nature of possible freeze-damage and the cause for bile sensitivity after freezing. Recovery of bile resistance of freeze-damaged *L. acidophilus* cells was complete in the presence of penicillin and chloramphenicol, which indicated that the repair of freeze-injury was

not dependent on the synthesis of peptidoglycan and protein. The inability of some (40%) of the cells to recover in the presence of rifampin and 2,4-dinitrophenol could be due to the need for RNA synthesis and ATP to repair freeze-injury. Freeze-injured *Escherichia coli* and *Salmonella anatum* are known to synthesize ATP to repair injury and to regain resistance to bile salts.

In the study by Brennan et al. (1986), *L. acidophilus* cells surviving freeze drying and vacuum drying became sensitive to oxgall, lysozyme and NaCl, and permeable to orthonitrophenol β-galactoside. They suggested that the sensitivity to oxgall and lysozyme could be due to damage to the cell wall, whereas the sensitivity to NaCl and permeability to orthonitrophenol could be due to damage to the cytoplasmic membrane. Scanning and transmission electron microscopy of the damaged cells indicated loss of some cell surface, cell wall, and cell membrane material, but to what extent these losses were due to the freeze drying and vacuum drying treatments was not clear.

Kole and Altosaar (1984) obtained a strain of *Leuconostoc oenos* with improved bile tolerance by subculturing it in gradually increasing concentrations of bovine bile. This strain was resistant to 1.0% bovine bile whereas there was previously a 90% reduction of growth in media supplemented with 0.5% bovine bile and practically no growth in media supplemented with greater than 0.5% bovine bile. The bile resistant *Leuconostoc oenos* strain was 20% more viable after lyophilization than strains not resistant to bovine bile. Kole and Altosaar (1984) observed changes in the fatty acid composition of the bile resistant cells, which contained more dodecanoic, hexadecanoic, and octadecanoic acids than the bile sensitive cells. The bile sensitive cells contained more tetradecanoic and hexadecanoic acids than the bile resistant cells.

Cells of both strains contained high percentages of C-19 cyclopropane fatty acid, which Kole and Altosaar (1984) hypothesized to be the influenced by the Tween 80 in the medium.

Noh and Gilliland (1993) reported that the presence of 0.3% oxgall increased the β-galactosidase activity of all five *L. acidophilus* strains tested. One of the two least bile tolerant *L. acidophilus* strains (ATCC 4356) had the highest activity of β-galactosidase, both in the presence and absence of bile, compared with the other strains. This illustrates how several factors must be taken into consideration when choosing a probiotic organism. Noh and Gilliland (1993) suggested that the increase of β-galactosidase activity in the presence of oxgall was a result of enhanced cellular permeability caused by oxgall, which would permit more substrate to enter the cells to be hydrolyzed.

2.5.3 Effect of colony morphology on bile tolerance

Two types of colonies of *L. acidophilus* were reported by Klaenhammer and Kleeman (1981). The rough R type of colony was described as large, irregular, flat to umbonate, dry and granular, with a matte surface and mottled opacity. The smooth S type of colony was described as smaller, circular, convex, moist and creamy, with a smooth edge and glistening, translucent appearance. The R and S colonies were isolated by picking the roughest and smoothest colonies several times onto MRS agar until the R and S colony types stabilized. Kopeloff (1934) observed many colonies of *L. acidophilus* whose morphology was somewhere between the R and S colony types. Klaenhammer and Kleeman (1981) found a correlation between the colonial

morphology of *L. acidophilus* and its bile sensitivity. The R type *L. acidophilus* culture was sensitive to 0.6% bile, whereas the S type was resistant to 1% bile.

At the cellular level, Klaenhammer and Kleeman (1981) described the R colonies as having a mixture of long and short rods in long chains and filaments, often observed as a tangled mass of cells. The S colonies were described as having homogeneous and individual short rods. They observed bleb-like protrusions from the cell wall of the type cells of *L. acidophilus* (RL8K), but rarely in the S type cells. An explanation for the greater sensitivity of R type colonies to bile could be that in the elongated cells more sites are exposed to surface-active agents such as bile salts (Kleeman and Klaenhammer, 1981). The in vitro filamentous growth, resulting in R colonies, probably occurs in growth conditions that hinder proper cell multiplication (Kleeman and Klaenhammer, 1981). This emphasizes the importance of proper medium in the isolation and propagation of probiotic strains.

It is not clear what colony type exists in the intestines and thus it is difficult to know what colony type would be the most suitable for probiotic products (Klaenhammer and Kleeman, 1981). Normally, *L. acidophilus* strains have been isolated from feces as R type colonies, with S type colonies appearing during laboratory propagation, probably as an adaptation to in vitro conditions. The occurrence of the S type colony has been associated with resistance to environmental stress.

Kleeman and Klaenhammer (1981) listed several environmental factors that have been reported to influence the colonial morphology of lactobacilli: acetate, fatty acid esters, salt, Tween 80, sodium oleate, antibiotics with surfactant properties in the growth medium, pH, incubation temperature, oxygen tension, total CO_2 in the

atmosphere, and dryness of agar surfaces. Lack of deoxyribonucleosides, vitamins, and divalent cations have been found to promote filamentous growth of lactobacilli. There is also naturally occurring heterogeneity in lactobacilli, which is observed when a pure isolate results in a heterogeneous culture, on a single complex medium, under stable environmental conditions, e.g. this is what Klaenhammer and Kleeman (1981) observed with *L. acidophilus* (RL8K) on MRS agar. In conclusion, Klaenhammer and Kleeman (1981) reported that the various colonial and cellular morphologies of lactobacilli, whether due to naturally occuring heterogeniety or due to environmental factors, can have an effect on the bile tolerance of lactobacilli.

2.6 USE OF BILE IN SELECTIVE BACTERIAL GROWTH MEDIA

Bile is used for selective enumeration, isolation and propagation of enteric organisms, especially coliform bacteria and other enteric bacteria such as *Salmonella* and *Shigella*, in media such as Brilliant Green Bile agar, Brilliant Green Lactose Peptone Bile 2%, Desoxycholate Lactose agar, MacConkey agar, Salmonella Shigella agar, and Violet Red Bile agar, for the bacteriological examination of water, foods and dairy products (Difco, 1984). Depending on the medium, either oxgall, which is dehydrated fresh bile (0.00029 to 2%), a mixture of bile salts (0.15 to 0.85%), or individual bile salts such as deoxycholic acid (0.05% in Desoxycholate lactose agar) is added as a selective agent. It is interesting to note that in Littman agar, for the isolation of fungi, oxgall is used to restrict the spreading of fungal colonies (Difco, 1984).

Gilliland and Speck (1977b) supplemented LBS agar with oxgall to 0.5, 0.10, and 0.15% concentration for selective enumeration of bile resistant lactobacilli. They

reported that the use of sodium taurocholate, instead of oxgall, resulted in comparable results, but that deoxycholate was precipitated, due to the acidity of LBS agar, and thus could not be used. When LBS agar, supplemented with oxgall, was tested by Gilliland and Speck (1977b), the two *L. bulgaricus* strains and the one *L. lactis* strain used, which are normally not isolated from the intestines, were not able to grow in LBS agar containing 0.15% oxgall, and the *L. lactis* strain and one of the *L. bulgaricus* strains were not able to grow in LBS agar containing 0.05% oxgall. All seven of the other lactobacilli (*L. acidophilus*, *L. brevis*, *L. casei*, *L. plantarum*, and *L. fermentum*), which have been isolated from intestinal sources, grew well in the presence of 0.15% oxgall. On the basis of these results, Gilliland and Speck (1977b) proposed that LBS agar, supplemented with 0.15% oxgall, is suitable for enumeration of bile resistant intestinal lactobacilli. They noted that when sampling the lactobacilli in some, especially dried, products, the incubation time had to be increased to 5 days from 3 days due to the apparent injury and resulting slow growth of the lactobacilli. To make the test for bile tolerance more sensitive, Gilliland et al. (1984) increased the concentration of oxgall to 0.3%, at which concentration each strain would be at least partially inhibited. Gilliland and Speck (1977b) preferred to use the agar count method to measure bile tolerance as a more reliable method than the broth method, but Gilliland et al. (1984) recommended the broth method because it would allow the comparison of the growth rates of bacteria in the presence of bile. Noh and Gilliland (1993) used the time required for the adsorption (620 nm) to increase by 0.3 units as the criterion for bile resistance of *L. acidophilus* in peptonized milk nutrient broth with 0.3% oxgall.

Floch et al. (1972) observed heavy turbidity of *Bacteroides* sp. grown in the presence of bile, but found very few viable organisms in the culture. The turbidity appeared to

be due to precipitation of bile salts. Similar observations were made by Klaver and Van der Meer (1993), who reported that the strains of *L. acidophilus*, *L. casei*, and *Bifidobacterium bifidum*, which deconjugate bile salts, showed lower biomass concentrations after incubation in the presence of oxgall compared with the concentration of biomass observed in MRS medium without oxgall.

Dashkevicz and Feighner (1989) developed an agar plate assay to detect bile salt hydrolase activity in lactobacilli. Detection of bile salt hydrolase activity was based on observations of *Lactobacillus* colonies grown on MRS or Rogosa SL agar supplemented with 0.5% TDCA. The bile deconjugating activity manifested itself as either the formation of halos of precipitated bile around colonies or as the formation of opaque granular white colonies. Both phenomena were related to the lower pKa values of the conjugated bile acids (pKa 1.9) compared with the higher pKa values of the free bile acids (pKa 5.0). According to these pKa's, free bile acids were protonated and precipitated at the end pH values of *Lactobacillus* fermentation, but taurine conjugates stayed completely ionized and remained in solution. The choice of the correct bile acid in relation to the pKa is important so that the bile acid will only precipitate when deconjugation occurs. Dashkevicz and Feighner (1989) saw possibilities for the use of this medium in the rapid screening of bile salt hydrolase-deficient mutants, thereby using bile salt hydrolase activity as a genetic marker.

2.7 SAFETY CONSIDERATIONS

Safety considerations are a very important aspect in choosing a probiotic organism. The focus of this section will be on the clinical importance of *Lactobacilus* sp. Lactobacilli have been consumed in food for thousands of years and are organisms

that in terms of United States Food and Drug Administration's regulations are "generally recognized as safe" (GRAS) (Havenaar et al., 1992). This terminolgy does not exist in the Canadian legislation. Various lactobacilli form part of the indigenous oral, intestinal and vaginal microflora of humans. However, there have been occasions when lactobacilli have been implicated in clinical infections. Aguirre and Collins (1993) and Gasser (1994) wrote extensive reviews on the involvement of lactic acid bacteria (LAB) in clinical infections. There is increasing evidence that LAB can act as opportunistic pathogens, primarily in cases where patients have underlying diseases. In 1938, a case of *Lactobacillus* endocarditis was reported (Gasser, 1994), and since then there have been occasional reports of serious infections possibly associated with *Lactobacillus* sp. (Aguirre and Collins, 1993; Gasser, 1994).

Lactobacillus sp. have been implicated in cases of endocarditis, but the occurrence of this is very low. The onset of disease was often preceded by tooth decay or a dental procedure, which suggests that the source of the *Lactobacillus* infection was often the oropharynx. According to the reviews by Aguirre and Collins (1993) and Gasser (1994), the *Lactobacillus* sp. most often involved in endocarditis is *L. rhamnosus* (*L. casei* subsp. *rhamnosus*). *L. casei* and *L. plantarum* are often associated with clinical infection. In a few cases of endocarditis, the *L. acidophilus* group was involved. These cases either had a predisposing cause or a cause that was not mentioned. In addition, in two cases of endocarditis, the presence of *L. acidophilus* was also suspected. Lactobacilli have also been involved in local infections such as chest, digestive tract, urinary tract and throat infections as well as meningitis. Again, there was an underlying disease or predisposing condition.

The involvement of LAB in bloodstream infections is extremely rare (Gasser, 1994). According to statistical data in France (Réseau National de Santé Publique), there was an average frequency of 0.101% for the isolation of *Lactobacillus* in bloodstream infections from 1988 to 1990. A similar frequency was found for patients tested in Vancouver, Canada (Roberts et al., 1991). Some of the *Lactobacillus* sp. involved were *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, and *L. jensenii*. *L. delbrueckii* subsp. *bulgaricus* was also involved in one case of bloodstream infection. In all of these cases the patients had an underlying disease that probably represented a predisposing condition for infection. In the case of the *L. delbrueckii* subsp. *bulgaricus* infection, the patient had leukemia as an underlying disease.

The reason for the apparent increased reports of lactobacilli as opportunistic pathogens may be due to improved detection techniques (media and identification techniques). With few exceptions, in the cases reviewed by Aguirre and Collins (1993) and Gasser (1994) the patient was immunocompromised due to extreme age, underlying disease or immunosuppressive therapy (antibiotics, chemotherapy, etc.). Certain lactobacilli have been known to cause dental caries, but this has not been a point of concern. Considering the fact that lactobacilli are ubiquitous in the human body, the frequency of occurrence of *Lactobacillus* infections is extremely low. There is no clear evidence of the presence of virulence factors in *Lactobacillus* sp.

The proper choice of a probiotic strain is extremely important. For example, Aguirre and Collins (1993) cast doubt on the use of enterococci as probiotics because of their intrinsic and acquired resistance to antibiotics. Both Aguirre and Collins (1993) and Gasser (1994) concluded that there already was a breakdown of host defences due to age, underlying disease or therapeutic treatment in the cases where LAB acted as

opportunistic pathogen. Aguirre and Collins (1993) and Gasser (1994) also concluded that the use of lactic acid bacteria in food fermentations is safe. This could likely be extended to orally administered probiotic products as well; however, great care should be taken in selecting a safe and effective probiotic organism.

3 MATERIALS AND METHODS

3.1 BACTERIAL CULTURES

The strains of *Lactobacillus* spp. used in this study are listed in Table 1.

Stock cultures of the *Lactobacillus* strains were maintained at -70°C in MRS broth containing 8% dimethyl sulfoxide (DMSO) or 20% glycerol as cryoprotectants. The cultures were propagated twice (1% inoculum) in fresh MRS broth prior to use in experiments and were subcultured a maximum of seven times before new working cultures were prepared. All *Lactobacillus* strains were incubated at 35°C.

3.2 CULTURE MEDIA

Sterile Lactobacilli MRS broth (Difco Laboratories, Detroit, MI, U.S.A.) and agar (Difco Laboratories, Detroit, MI, U.S.A.) (1.5% added agar) were used to propagate the *Lactobacillus* cultures. For bile tolerance testing, ox-bile (Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK) was added to MRS broth or agar in various concentrations (0 to 10% w/v) before autoclaving (20 min, 121°C), referred to as bMRS broth and bMRS agar, respectively, with the percentage of bile indicated in front of the name of the medium, e.g. 0.15% bMRS broth refers to MRS broth containing 0.15% ox-bile. Precipitation of bile acids was observed in sterile tubes of bMRS after storage at 4°C for one to two weeks, but this turbidity disappeared when the medium was warmed to room temperature. Sterile 1% bMRS became turbid after 3 months at room temperature. No precipitation was observed after storage of sterile bile media at 35°C for one week. Because of these observations, fresh bMRS and bMRS agar was prepared before each experiment.

Table 1. Strains of *Lactobacillus* spp. used in the study.

Strain	Comments
L. acidophilus ATCC 33198[a]	Originally isolated from hog small intestine. Included in *L. acidophilus* subgroup A3, which was recognized as the same as the existing species *L. amylovorus* (Fujisawa et al., 1992).
L. acidophilus ATCC 43121[a]	Originally isolated from pig rectum. Cholesterol assimilation (Gilliland et al., 1985)
L. acidophilus BG2-FO4	Obtained from T.R. Klaenhammer (Department of Food Science, North Carolina State University, Raleigh, NC).
L. acidophilus F	Isolate from French milk product 'Forza'.
L. acidophilus N2	University of Alberta Food Microbiology Laboratory culture collection.
Lactobacillus GG ATCC 53103[a]	Originally isolated from human feces. Resembles *L. casei* subsp. *rhamnosus* (Silva et al., 1987).
L. bulgaricus J6, J9, J10, and J11	Obtained from Dr. J. Mostert (Research Institute for Animal and Dairy Science, Irene 1675, S. Africa).
L. delbrueckii subsp. *bulgaricus* ATCC 11842[a]	*Lactobacillus delbrueckii* subsp. *bulgaricus* type strain (Kandler and Weiss, 1986).
L. delbrueckii subsp. *bulgaricus* ATCC 7995[a]	Production of cheese.

a ATCC = American Type Culture Collection

For the curing experiments, various amounts of filter-sterilized novobiocin (Na-salt, Sigma Chemical Company, St. Louis, MO, U.S.A.) solution (1 mg/ml of Milli-Q water) was added aseptically to the medium after autoclaving. The 11% reconstituted skim milk (Lucerne, Canada) used for coagulation testing was autoclaved isothermally (30 min, 100°C).

3.3 BILE TOLERANCE

The bile tolerance of the *Lactobacillus* test strains was evaluated in three different ways: (1) For preliminary screening, 16 to 20 h old cultures of the *Lactobacillus* test strains were inoculated (0.1%) into 0 to 10% bMRS broth and incubated at 35°C. Growth was monitored visually after 24, 48, and 72 h of incubation; (2) Growth on bMRS agar was studied by preparing a dilution series (0 to 10^{-6} dilution) of the 16 to 20 h old cultures of the *Lactobacillus* test strains in 0.1% peptone water, and inoculating 20 µl drops onto 0 to 10% bMRS agar. The plates were incubated in anaerobic jars at 35°C for 72 h. Colonies were counted after 24, 48, and 72 h; (3) The viability of the *Lactobacillus* test strains in bMRS broth was determined in three separate trials. The 16 to 20 h old cultures of the test strains were inoculated (0.1%) into 0, 0.15, 0.3, 0.6, 1, 2, 2.5, 3, 4, and 5% bMRS broth, and incubated at 35°C for up to 72 h. The bMRS culture tubes were sampled at various time points to determine the number of cfu/ml in the tube, which was done by preparing a dilution series (0 to 10^{-6} dilution) in 0.1% peptone water, and inoculating 20 µl drops onto MRS agar. The plates were incubated in anaerobic jars at 35°C for 72 h. Colonies were counted after 24, 48, and 72 h of incubation. During one of the trials, an automated microplate reader (Model EL 309, Bio-Tek Instruments, Inc., Burlington,

VT) was used for monitoring growth in bMRS broth, in addition to determining the viable counts as described above. After mixing the test culture on a vortex mixer, 100 µl of the culture, or a dilution of it made into bMRS broth with the appropriate concentration of bile, was pipetted into a well of a microwell plate (Nunclon, Nunc, Denmark). This was done in duplicate for each culture. Sterile bMRS broth with appropriate concentration of bile was used as a blank. Before reading the absorbance at 660 nm, the cultures in the wells of the plate were mixed by using a microtiter plate shaker. The absorbance of the appropriate blank was subtracted from the absorbance of the sample.

3.4 SELECTION FOR INCREASED BILE TOLERANCE

The procedure used to increase the bile tolerance of *L. bulgaricus* strains J9 and ATCC 11842 was based on the enrichment method used for *Leuconostoc oenos* by Kole and Altosaar (1984). Strains were tested for the highest bile concentration that allowed growth. This was determined visually. The *L. bulgaricus* strains J9 and ATCC 11842 were inoculated (0.1% inoculum) into 6 ml of 0.1% bMRS broth and incubated at 35°C for 72 h. Cells were collected aseptically by centrifuging in small batches for 1.5 min in an Eppendorf 5415 C microcentrifuge (Eppendorf, Netheler, Hinz GmbH, Hamburg, Germany) at high speed and resuspended in 500 µl of fresh 0.2% bMRS broth. This cell suspension was used to inoculate a fresh tube (6 ml) of bMRS broth (0.2%) with 1% inoculum. The culture was incubated at 35°C for 72 h. The enrichment procedure was repeated with increasing concentrations up to 1.6% bMRS broth.

3.5 PHYSIOLOGICAL TESTS ON EXPERIMENTAL CULTURES

3.5.1 Fermentation profiles

Fermentation profiles of the experimental strains were determined on carbohydrate fermentation agar as described by Shaw and Harding (1985) using the following substrates: amygdalin (Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.A.), cellobiose (Sigma Chemical Company, St. Louis, MO, U.S.A.), D-fructose (Fisher Scientific, Fair Lawn, NJ, U.S.A.), galactose (Sigma Chemical Company, St. Louis, MO, U.S.A.), glucose (BDH), lactose (Fisher Scientific, Fair Lawn, NJ, U.S.A.), maltose (Pfanstiehl Laboratories), mannitol (Fisher Scientific, Fair Lawn, NJ, U.S.A.), D-mannose (Difco Laboratories, Detroit, MI, U.S.A.), raffinose (Difco Laboratories, Detroit, MI, U.S.A.), ribose (Sigma Chemical Company, St. Louis, MO, U.S.A.), salicin (Sigma Chemical Company, St. Louis, MO, U.S.A.), sucrose (BDH Inc., Toronto and braches), trehalose (Sigma Chemical Company, St. Louis, MO, U.S.A.) and D-xylose (BDH Inc., Toronto and braches). The basal medium was prepared as follows: yeast extract (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, U.S.A.) 6 g; trypticase peptone (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD, U.S.A.) 15 g; cysteine-HCl (Sigma Chemical Company, St. Louis, MO, U.S.A.) 0.2 g; Tween 80 (Anachemia, Montreal/ Mississauga/ Rouses Point) 1 g; chlorophenol red (Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.A.) 40 mg; $MgSO_4 \times 7\ H_2O$ (Fisher Scientific, Fair Lawn, NJ, U.S.A.) 0.2 g; $MnSO_4 \times 4H_2O$ (AnalaR, BDH Chemicals, Toronto/ Montreal/ Vancouver) 0.5 g; agar (Difco Laboratories, Detroit, MI, U.S.A.) 12 g; in 1000 ml of distilled water, and pH was adjusted to 6.4. The medium was dispensed in 19 ml aliquots in screw cap tubes and autoclaved (20 min, 121°C). After the autoclaved medium had cooled to 45° C, 1 ml

of 10% filter sterilized carbohydrate solution was added and gently mixed into the molten agar and dispensed into petri dishes. A control plate without added carbohydrate was included in the test. The carbohydrate fermentation test plates and the contol plate were inoculated with an inoculator (Cathra), that spotted 12 cultures per plate. Inoculated plates were incubated at 35° C in an anaerobic jar, examined for growth after 24 and 48 h of incubation. A yellow zone around the colony indicated fermentation of the substrate.

3.5.2 API identification strips

Fermentation profiles of selected strains of *Lactobacillus* sp. were determined by API 50 CHL identification strips (BioMérieux Vitek Inc., France) according to manufacturer's instructions. Before use, the purity of each strain was checked on MRS agar. For the API strip testing, overnight cultures were centrifuged at 7,710 x *g* for 10 min. The pellets were washed with sterile Milli-Q water, centrifuged as described above, and resuspended in 2 ml of Milli-Q water. This cell suspensio. was used to adjust the optical density (550 nm) of 5 ml of CHL medium (supplied in the kit) to 0.5 to 0.75. Twice the amount of cell suspension was used to inoculate 10 ml of CHL medium, which was used for inoculating the API test strips. The test strips were incubated at 35°C, and the results were checked after 3, 6, 24, and 48 h of incubation. The strains were identified by matching the fermentation profiles to existing profiles in a API database.

3.5.3 Coagulation of skim milk

Growth in skim milk was tested by inoculating the test strains (0.1% inoculum) into 11% reconstituted skim milk and incubating at 35°C. After 24 h of incubation the inoculated skim milk samples were checked visually for coagulation.

3.6 PLASMID ISOLATION

Small scale plasmid isolation was used for preliminary testing of the strains of *Lactobacillus* sp. from the curing experiments. Small scale plasmid isolation was first performed for strains BG2-FO4, ATCC 33198 and ATCC 53103 according to the method by Anderson and McKay (1983) with some modifications, but later the method for *Lactococcus* and *Lactobacillus* spp. developed by O'Sullivan and Klaenhammer (1993) was used for strains ATCC 43121 and J9. Large scale plasmid extraction was performed according to the method by Anderson and McKay (1983) with some modifications.

3.6.1 Plasmid isolation according to a modified version of the method by Anderson and McKay (1983)

The quantities of reagents used in the large scale plasmid isolation are shown in brackets.

In preparation for the small scale plasmid isolation, the experimental strain was inoculated (1% inoculum) into 10 ml of MRS broth and incubated for 12 h at 35°C. A 4-ml amount of the 12-h culture was centrifuged for 3 min at 17, 300 x *g* at 4°C. The cells were resupended in 10 ml of fresh MRS broth and incubated for 2 h at 35°C,

after which the cells were harvested by centrifugation as described above. The cells were washed with cold sterilized Milli-Q water, resuspended in 1 ml of cold (4°C) sterilized Milli-Q water, and placed in an Eppendorf tube. The cells were centrifuged for 1.5 min in an Eppendorf 5415 C microcentrifuge at high speed, and the supernatant was removed.

In preparation for the large scale plasmid isolation, the experimental strain was inoculated (0.1% inoculum) into 100 ml of MRS broth and incubated at 35°C for 18 to 22 h. The cells were harvested by centrifugation at 13, 200 x *g* for 12 min at 4°C, and washed with cold sterilized Milli-Q water.

The cell pellet was resuspended in 380 µl (30 ml) of 6.7% sucrose, 50 mM Tris-HCl, 1 mM EDTA (pH 8.0).

In small scale isolation, the sample was tempered for 2 min in a 37°C waterbath. A 96.5 µl quantity of lysozyme solution [10 mg lysozyme (Sigma Chemical Company, St. Louis, MO, U.S.A.) in 25 mM Tris-HCl (pH 8.0)], and 4 µl of RNAase A [Sigma Chemical Company, St. Louis, MO, U.S.A.; 10 mg/ml stock prepared according to Sambrook et al. (1989); stored at -20°C] was added, and the sample was mixed at low speed on a vortex mixer.

In large scale isolation, 7.5 ml of 25 mM Tris (pH 8.0) was added, after which the pH was adjusted to pH 8.5 to 8.6. A 0.1125 g quantity of lysozyme was added to the tube as dry powder.

After incubation for 7 min (20 min) at 37°C, 50 µl (3.75 ml) of 0.25 M EDTA in 50 mM Tris-HCl (pH 8.0) was added. Then 28 µl (2.25 ml) of 20% SDS (w/v) in 50 mM Tris: 20 mM EDTA (pH 8.0) was added and mixed immediately. After incubation for 10 min (25 min) at 37 °C the sample was mixed on a vortex mixer for 30 s at high speed.

In small scale, 28 µl of 3 N NaOH was added. In large scale, the mixture was adjusted to pH 12.2.

The sample was placed on ice, and was mixed several times during 10 min incubation. A 50 µl (3.90 ml) quantity of 2 M Tris-HCl (pH 7.0) was added and mixed by gentle inversion of the tube. The mixture was held at room temperature for 5 min. Before that, in large scale, the mixture was adjusted to pH 7.0 to 7.5.

A 72-µl (5.7-ml) amount of 5 M NaCl was added, mixed by inversion, placed on ice for 30 min, centrifuged for 10 min in an Eppendorf 5415 C microcentrifuge at high speed (for 15 min at 13,200 x *g*) at 4°C, and the supernatant was decanted into a clean tube. For deproteinization of the supernatant, 400 µl (60 ml) of 3% NaCl-saturated phenol (BDH) was added and mixed by inversion. In large scale, 60 ml of a (24:1) mixture of chloroform and isoamylalcohol, was also added and mixed on a gently rotating platform (Red Rotor model PR70, Hoefer Scientific Instruments, San Fransisco) for 10 min and allowed to stand for 5 min.

Samples were centrifuged as described above. The upper aqueous layer was transferred into a clean tube. In large scale, deproteinization was repeated as described above.

A 700 µl (60 ml) amount of a (24:1) mixture of chloroform and isoamylalcohol was added and the deproteinized sample was mixed by inversion and centrifuged as described above. The upper layer was transferred into a clean tube, and 700 µl (2 vol) of cold 95% ethanol was added to precipitate the DNA at 4°C overnight. The DNA was recovered by centrifugation for 12 min in a microcentrifuge at high speed (for 20 min at 13,200xg) at 4°C. The supernatant was carefully removed. In large scale, the DNA was washed with 70% ethanol.

The DNA pellet was dried in a vacuum dryer (SpeedVac SC100, Savant Instruments Inc., Farmingdale NY, U. S. A.). The dried pellet was resuspended in 25 µl (1 ml) of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). In large scale, 6 µl of RNAase A was added at this point. DNA was separated by gel electrophoresis as described in section 3.6.4.

3.6.2 Small scale plasmid isolation according to O'Sullivan and Klaenhammer (1993)

A 6 ml amount of an overnight cultures of the strains in question was centrifuged at 5, 900 x *g* for 15 min. The cell pellet was resuspended in 200 µl of 25% sucrose containing 30 mg of lysozyme per ml. The suspension was transferred into an Eppendorf tube and incubated at 37°C for 15 min. A 400 µl quantity of alkaline SDS solution (3% sodium dodecyl sulfate in 0.2 N NaOH) was added and mixed without delay. After incubation for 7 min at room temperature, 300 µl of ice-cold 3 M sodium acetate (pH 4.8) was added and mixed immediately, after which the tube was centrifuged for 15 min at maximum speed in an Eppendorf 5415 C microcentrifuge at

4°C. The supernatant was transferred into a clean Eppendorf tube, and 650 µl of isopropanol (room temperature) was added and mixed well. The tube was centrifuged as described above. All of the liquid was removed and the pellet was resuspended in 320 µl of sterile Milli-Q water. A 200-µl amount of ammonium acetate containing ethidium bromide (0.5 mg/ml) was added and mixed, after which 350 µl of phenol/chloroform solution was added. The sample was centrifuged for 5 min at maximum speed in an Eppendorf 5415 C microcentrifuge at room temperature. The upper phase was transferred into a clean Eppendorf tube to which 1 ml of ethanol (-20°C) was added After precipitation overnight at -20°C, DNA was recovered by centrifuging for 15 min at maximum speed in an Eppendorf 5415 C microcentrifuge at 4°C. The pellet was washed in 70% ethanol at room temperature. All of the liquid was removed and the pellet was dried for 5 min in a vacuum dryer. The dried pellet was resuspended in 20 µl of Tris-EDTA (TE) buffer (pH 8.0) containing 0.1 mg/ml RNAseA (See p. 5). The DNA sample was separated by gel electrophoresis as described later in section 3.6.4.

3.6.3 Purification of plasmid DNA

The DNA samples from large scale extraction were purified by equilibrium centrifugation in continuous cesium chloride - ethidium bromide gradients according to Sambrook et al. (1989) with some modifications. The DNA samples, dissolved in TE buffer (pH 8.0), were collected from one or more large scale plasmid isolations and pooled. For every milliliter of DNA solution, 1 g of solid cesium chloride was added and mixed gently until dissolved. To 7 ml of DNA - cesium chloride solution, 0.4 ml of ethidium bromide (Sigma Chemical Company, St. Louis, MO, U.S.A.; 10 mg/ml of TE buffer) was added. The density gradients were centrifuged in 12 ml

Beckman (U.S.A.) polyallomer tubes at 45, 000 x *g* for 20 to 24 h at 20°C in a Beckman Ti.70.1 rotor. The DNA bands were examined under UV light and collected with a pasteur pipette and transferred to a clean plastic tube. The upper band consisted of linear bacterial (chromosomal) DNA and nicked (open) circular plasmid DNA, and the lower band contained closed circular plasmid DNA.

Ethidium bromide was removed from DNA by extraction with an equal amount of isoamyl alcohol. The sample was mixed gently by inversion, protected from light, after which the lower aqueous phase was transferred to a clean tube. The extraction was repeated until all of the pink colour had disappeared. Cesium chloride was removed by dialysis against TE buffer (pH 8.0) in Spectra/Por Molecularporous Membrane Tubing (# 1, molecular weight cut off 6000 to 8000, Spectrum Medical Industries, Houston, Texas), prepared according to Sambrook et al. (1989). The dialysis buffer was changed twice during the first half hour, after which dialysis was continued for 18 to 24 h. The dialyzed sample was recovered from the dialysis tubing with a pasteur pipette. DNA was separated by gel electrophoresis.

3.6.4 Gel electrophoresis

DNA was separated by gel electrophoresis on 0.6% agarose (GIBCO BRL, Gaithesburg MD, USA) gel in 0.5 x Tris-borate (TBE) buffer in a horizontal gel electrophoresis unit (HG-12, Tyler Research Instruments, Canada). The 0.5 x TBE buffer was diluted from a 5 x concentrated TBE buffer stock (54 g Tris base; 27.5 g boric acid; 20 ml 0.5 M EDTA (pH 8.0); made up to 1 l of stock solution with Milli-Q water). Ethidium bromide was included in the gel and in the buffer: for every 20 ml of agarose gel and buffer, 1 µl of ethidium bromide solution (10 mg/ml) was added. The DNA samples (5 to 20 µl) from the small and large scale plasmid extractions, and

cesium chloride extraction, were loaded into the wells of the gel. Before loading, gel tracking dye (1 to 4 µl) was added to the samples and the *Escherichia coli* V517 plasmid size marker (Macrina et al., 1978). The loaded gel was exposed to a constant voltage of 100 V, supplied from electrophoresis power supply (EPS 500/400, Pharmacia). After 3 h, the gel was removed and viewed under UV light at 300 nm with a Foto UV 300 DNA Transilluminator (Fotodyne Inc., New Berlin, WI). The plasmids of *E. coli* V517 were used to estimate the sizes of the plasmid bands of the*Lactobacillus* test strains (Macrina et al., 1978).

3.7 PLASMID CURING

A combination of novobiocin and elevated temperature (45°C) was used to achieve curing of plasmids of the selected *Lactobacillus* strains. The minimum inhibition concentration (MIC) for novobiocin of the *Lactobacillus* strains used in the curing experiment was determined at 45°C (= $MIC_{45°C}$). The strains were inoculated into a tubes of MRS containing novobiocin, and incubated at 45°C in a waterbath. The highest concentration of novobiocin allowing growth was the $MIC_{45°C}$ of the strain in question.

The 12- to 14-h cultures were inoculated at 0.1% inoculum level into MRS broth containing novobiocin at the $MIC_{45°C}$ concentration for the specific strain, and incubated at 45°C in a water bath. The culture tube with the highest concentration of novobiocin displaying turbidity was screened for loss of bile tolerance.

Screening was done by differential colony counts on MRS agar with and without added bile. This was done at various stages of curing. Dilution series (0 to 10^{-6}

dilution) of the novobiocin cultures, made in 0.1% peptone water (Difco Laboratories, Detroit, MI, U.S.A.), was plated onto 0 and 5% bMRS agar and incubated in an anaerobic jar at 35°C for 48 h. When $\geq$ 2 log unit difference in cell count was observed between 0 and 5% bMRS agar, there was a good probability of finding a mutant strain, and colonies were picked from 0% bMRS agar and replica-plated onto 0 and 5% bMRS agar to check for loss of bile tolerance. The plates were incubated in anaerobic jars at 35°C and were examined for growth after 48, 72, and 96 h. Even longer incubation times were used for bMRS agar. In the absence of growth on 5% bile plate, the colony was picked from the 0% plate, subcultured into MRS broth and stored in MRS stabs for further testing.

4. RESULTS

4.1 INITIAL SCREENING

4.1.1 Screening of strains of *Lactobacillus* spp. for bile tolerance

Five strains of *L. acidophilus*, six strains of *L. delbrueckii* subsp. *bulgaricus*, and *Lactobacillus* GG were screened for bile tolerance in MRS broth and on MRS agar with various concentrations (0, 0.15, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10%) of bile. The results of the screening for bile tolerance are shown in Table 2. The population on MRS agar (without bile) is used as a reference for the results obtained in the presence of bile.

From the results presented in Table 2, it can be seen that the strains of *L. delbrueckii* subsp. *bulgaricus* were more sensitive to bile than the strains of *L. acidophilus* or *Lactobacillus* strain GG and were inhibited by lower concentrations of bMRS agar than the other strains tested. Populations of *L. acidophilus* strains ATCC 33198 and F, and *Lactobacillus* GG strain ATCC 53103 were not affected by 10% bile. Based on the results shown in Table 2, strains ATCC 33198, BG2-FO4, ATCC 43121, and ATCC 53103 were chosen as the strains of *Lactobacillus* spp. for the plasmid curing experiments because of their high level of bile tolerance. *L. delbrueckii* subsp. *bulgaricus* strains J9 and ATCC 11842 were selected as bile sensitive strains for use in experiments for improving their bile tolerance.

Table 2. Initial screening of selected *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *Lactobacillus* GG strains for growth in bMRS broth and on bMRS agar after 72 h incubation at 35°C. Counts are the mean of two determinations.

Strain	Max% bile in broth with growth (%)	Population on MRS agar (log cfu/ml)	Max % bile in agar without affecting population[a] (%)	(log cfu/ml)	Max % bile in agar supporting growth (%)	(log cfu/ml)
L. acidophilus						
ATCC 33198	10[b]	9.02	5	9.13	10[b]	6.54
F	10[b]	8.93	10[b]	8.85	10[b]	8.85
N2	10[b]	8.68	10[b]	8.29	10[b]	8.29
BG2-FO4	10[b]	8.57	6	7.52	10[b]	3.70
ATCC 43121	10[b]	9.09	10[b]	8.72	10[b]	8.72
Lactobacillus GG						
ATCC 53103	10[b]	9.06	10[b]	8.74	10[b]	8.74
L. bulgaricus						
J6	6	8.54	0	8.54	1	4.00
J9	0.15	8.26	0	8.26	5	<2.70[c]
J10	8	8.72	0	8.72	3	4.00
J11	5	8.64	0.15	8.52	5	4.30
ATCC 11842	0.15	8.67	0	8.67	0.15	<2.70[c]
ATCC 7995	1	7.79	0.15	7.15	3	3.70[c]

a Compared with the population on MRS agar.
b The highest concentration of bMRS tested.
c Marginal growth

4.1.2 Characterization of the experimental strains by fermentation profiles

The experimental strains (except for strain J6) were characterized for their fermentation profiles by API 50 CHL test strips. The results for strains that utilized the energy sources for growth are presented in Table 3. None of the strains used glycerol, erythritol, D-xylose, L-xylose, adonitol, β-methyl-xyloside, L-sorbose, rhamnose, α-methyl D-mannoside, α-methyl D-glucoside, melibiose, inulin, xylitol, D-turanose, D-lyxose, D-fucose, D-arabitol, L-arabitol, 2-keto-gluconate, 5-keto-gluconate as an energy source in the API CH50 test. The fermentation profiles of strains J9, J10, ATCC 11842, ATCC 43121, ATCC 33198, and ATCC 53103 were also tested on carbohydrate fermentation agar (Table 4). The classification of the *Lactobacillus* strains based on the API testing is shown in Table 5.

The identification of some of the *Lactobacillus* strains by API 50 CHL tests differed from the original identification. Based on results from API 50 CHL test, *L. acidophilus* ATCC 43121 should be identified as *L. delbrueckii* subsp. *delbrueckii*, because of the negative test on maltose. However, based on the positive reaction on maltose plate test and the fermentation profiles for the 15 other tests, ATCC 43121 was confirmed as *L. acidophilus*, as it originally has been classified in the ATCC catalogue. According to Pot et al. (1994), there is substantial variation in the determination of the carbohydrate fermentation pattern depending on the procedures used. Even with highly standardized, commercially available, miniaturized systems, such as API 50 CHL, considerable interlaboratory variation occurs.

Table 3. Fermentation profiles of the experimental strains according to API 50 CHL test strips. Results shown are based on one test.

	ATCC 33198	ATCC 43121	BG2-FO4	F	N2	ATCC 53103	J9	J10	J11	ATCC 11842	ATCC 7995
Sor	-	-	-	-	-	+	-	-	-	-	-
D-ara	-	-	-	-	-	+	-	-	-	-	-
L-ara	-	-	+	-	-	-	-	-	-	-	-
Rib	-	-	-	-	-	+	-	-	-	-	-
Gal	+	-	+	+	+	+	-	+	+	-	-
D-glu	+	+	+	+	+	+	+	+	+	+	+
D-fru	+	+	+	+	+	+	+	+	+	+	-
D-mne	+	+	+	+	+	+	+	+	+	+	-
Dul	-	-	-	-	-	+	-	-	-	-	-
Ino	-	-	-	-	-	+	-	-	-	-	-
Man	-	-	-	+	-	+	-	-	-	-	-
Nag	+	+	+	+	+	+	-	+	+	-	-
Amy	-	-	+	+	+	+	-	-	-	-	-
Arb	-	-	+	+	-	+	-	-	-	-	-
Esc	+	+	+	+	+	+	-	-	-	-	-
Sal	+	-	+	+	+	+	-	-	-	-	-
Cel	+	-	+	+	+	+	-	-	-	-	-
Mal	+	-	+	+	+	-	-	-	-	-	-
Lac	-	-	+	+	+	-	+	+	+	+	-
Sac	+	+	+	+	+	+	-	-	-	-	-
Tre	-	-	+	+	+	+	-	-	-	-	-
Mel	-	-	-	-	-	+	-	-	-	-	-
D-raf	-	-	+	+	+	-	-	-	-	-	-
Starch	-	-	+	-	-	-	-	-	-	-	-
Glyg	-	-	+	-	-	-	-	-	-	-	-
B Gen	+	-	+	+	+	+	-	-	-	-	-
D-tag	-	-	-	-	-	+	-	-	-	-	-
L-fuc	-	-	-	-	-	+	-	-	-	-	-
Gnt	-	-	-	-	-	+	-	-	-	-	-

+	positive		
-	negative		
Sor	= sorbitol	Esc	= esculin
D-ara	= D-arabinose	Sal	= salicin
L-ara	= L-arabinose	Cel	= cellobiose
Rib	= ribose	Mal	= maltose
Gal	= galactose	Lac	= lactose
D-glu	= D-glucose	Sac	= saccharose
D-fru	= D-fructose	Tre	= trehalose
D-mne	= D-mannose	Mel	= melezitose
Dul	= dulcitol	D-raf	= D-raffinose
Ino	= inositol	Starch	= starch
Man	= mannitol	Glyg	= glycogen
Nag	= N-acetyl-glucosamine	β Gen	= β Gentiobiose
Amy	= amygdalin	D-tag	= D-tagatose
Arb	= arbutin	L-fuc	= L-fucose
		Gnt	= gluconate

Table 4. Fermentation profiles of selected *Lactobacillus* strains on carbohydrate fermentation agar.

	ATCC 33198	ATCC 43121	ATCC 53103	J9	J10	ATCC 11842	*L. ac.*[a]	*L. amy.*[b]	*L. delbr. bulg.*[c]	*L. delbr. delbr.* d	*L.* GG[e]
amygdalin	+	+	+	-	-	-	+	$+_w$	-	-	-
cellobiose	+	+	+	-	-	-	+	+	-	#	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+
galactose	+	+	+	-	+	-	+	+	-	-	nd
gluconate	-	-	+	-	-	-	-	-	-	-	nd
glucose	+	+	+	+	+	+	+	+	+	+	+
lactose	nd	+	-	+	nd	+	+	-	+	-	-
maltose	+	+	-	-	-	-	+	+	-	#	-
mannitol	+	-	+	-	-	-	-	-	-	-	+
mannose	+	+	+	+	+	+	+	+	-	+	+
raffinose	nd	+	-	-	nd	-	#	-	-	-	-
ribose	-	-	+	-	-	-	-	-	-	-	+
salicin	-	+	+	-	-	-	+	$+_w$	-	-	+
sucrose	+	+	-	-	-	-	+	+	-	+	-
trehalose	-	+	+	-	-	-	#	+	-	#	+
xylose	-	-	-	-	-	-	-	-	-	-	+

+	positive	$+_w$	positive to weak reaction
-	negative		
#	11 - 89% strains positive		
nd	not determined		

a *Lactobacillus acidophilus* fermentation profile according to Kandler and Weiss (1986).

b *Lactobacillus amylovorus* fermentation profile according to Kandler and Weiss (1986).

c *Lactobacillus delbrueckii* subsp. *bulgaricus* fermentation profile according to Kandler and Weiss (1986).

d *Lactobacillus delbrueckii* subsp. *delbrueckii* fermentation profile according to Kandler and Weiss (1986).

e *Lactobacillus* GG fermentation profile according to Silva et al., 1987.

Table 5. Identification of experimental strains by API 50 CHL testing.

Strain	Original identification	API
ATCC 33198	*Lactobacillus acidophilus*[a]	*Lactobacillus delbrueckii* [b]
ATCC 43121	*Lactobacillus acidophilus*	*Lactobacillus delbrueckii* subsp. *delbrueckii*
BG2-FO4	*Lactobacillus acidophilus*	*Lactobacillus acidophilus*
F	*Lactobacillus acidophilus*	*Lactobacillus acidophilus*
N2	*Lactobacillus acidophilus*	*Lactobacillus acidophilus*
ATCC 53103	*Lactobacillus* GG[c]	*Lactobacillus casei* subsp. *alactosus/pseudoplantarum*
J9	*Lactobacillus delbrueckii* subsp. *bulgaricus*	*Lactobacillus delbrueckii* subsp. *bulgaricus*
J10	*Lactobacillus delbrueckii* subsp. *bulgaricus*	*Lactobacillus helveticus*[b]
J11	*Lactobacillus delbrueckii* subsp. *bulgaricus*	*Lactobacillus helveticus*[b]
ATCC 11842	*Lactobacillus delbrueckii* subsp. *bulgaricus*	*Lactobacillus delbrueckii* subsp. *bulgaricus*
ATCC 7995	*Lactobacillus delbrueckii* subsp. *bulgaricus*	*Lactobacillus fructivorans*[b]

a Reclassified as *Lactobacillus amylovorus* (Fujisawa et al., 1992)

b Low discrimination

c Resembles *Lactobacillus casei* subsp. *rhamnosus* (Silva et al., 1987)

The experimental strains (except for strain ATCC 7995) were tested for their ability to coagulate skim milk within 24 h at 35°C. *L. acidophilus* strains F and N2, and *L. delbrueckii* subsp. *bulgaricus* strains J6, J9, J10, J11, and ATCC 11842 coagulated skim milk within 24 h. *L. acidophilus* trains ATCC 33198, ATCC 43121, and BG2-FO4, and *Lactobacillus* GG strain ATCC 53103 did not coagulate skim milk within 24 h.

4.1.3 Plasmid profiles of *Lactobacillus* test strains

The plasmid profiles were determined for four strains of *Lactobacillus* sp., that grew on 5% bMRS agar (See section 4.1.1). These strains were *Lactobacillus acidophilus* ATCC 43121, ATCC 33198, and BG2-FO4, and *Lactobacillus* GG ATCC 53103. In addition, the plasmid profile of bile sensitive strain J9 was determined. A schematic presentation of the plasmid profiles of the *Lactobacillus* test strains based on the small scale plasmid isolation is shown in Figure 1. No plasmid DNA bands were seen in the plasmid profile of strain J9.

The plasmid profile of strain ATCC 43121 had four bands: one DNA band at the chromosomal level, and three plasmid DNA bands (Fig. 1) (More information on the plasmid profile of strain ATCC 43121 is presented in Section 4.2.2). The plasmid profile of both strains ATCC 33198 and ATCC 53103 had two plasmid DNA bands, one of them at similar relative migration distance (Fig. 1). Strain BG2-FO4 had a plasmid profile with one large plasmid DNA band traveling above the chromosomal DNA band.

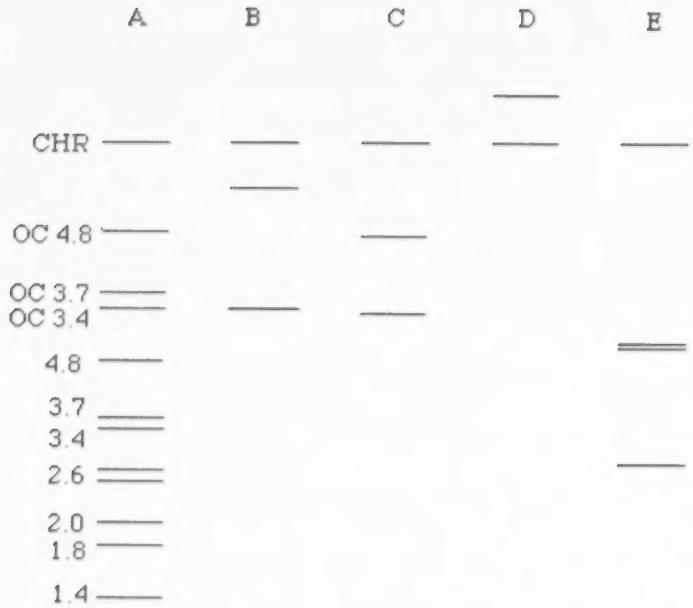

Fig 1. Schematic presentation of the plasmid profiles of *Lactobacillus* spp. strains *L. acidophilus* ATCC 33198 (B), *Lactobacillus* GG ATCC 53103 (C), *L. acidophilus* BG2-FO4 (D), and *L. acidophilus* ATCC 43121 (E), with the plasmids of *Escherichia coli* V517 (A) as the size marker. Sizes of plasmids are expressed in MDa. CH indicates chromosomal DNA and OC indicates open circular DNA.

4.2 EFFECT OF PLASMID CURING ON BILE TOLERANCE OF *LACTOBACILLUS* SPP.

4.2.1 Plasmid curing and screening for loss of bile tolerance

A combination of novobiocin and elevated temperature (45°C) was used to cure plasmids of four strains: *Lactobacillus* GG strain (ATCC 53103), and *L. acidophilus* strains ATCC 33198, ATCC 43121 and BG2-FO4. The data in Table 6 show the concentrations of novobiocin used, and the results of the differential counting on MRS and 5% bMRS agar that were used to screen for mutant colonies.

Because no growth of strains ATCC 33198 or BG2-FO4 was detected on 5% bMRS agar after novobiocin treatment, the chance of injury of these strains due to exposure to novobiocin was suspected (Table 6). After exposure of strain ATCC 53103 to novobiocin, differential counting showed no difference between its colony counts (cfu/ml) on MRS and 5% bMRS agar (Table 6). Counts of strain ATCC 53103 were reduced on MRS agar without bile after the novobiocin treatment indicating that the novobiocin treatment caused injury. Because of similar growth on both MRS agar and 5% bMRS agar, strain ATCC 53103 was excluded from further testing. Counts of strain ATCC 43121 on 5% bMRS agar were three log cycles less than counts on MRS agar (Table 6). This indicated a 1:1,000 chance of finding a strain that had lost its bile tolerance.

Based on the results of differential counting, further study was focused on *L. acidophilus* ATCC 43121. A total of 2,450 colonies of strain ATCC 43121 was

Table 6. Results of differential counting after curing of strains of *Lactobacillus* with novobiocin at 45°C.

Strain	Novobiocin µg/ml	# times subcultured	Count on MRS agar log cfu/ml	Count on 5% bMRS agar log cfu/ml
L. acidophilus ATCC 33198	0		9.23	9.13
	90	1	7.60	<1.70[a]
L. acidophilus ATCC 43121	0		9.06	8.78
	13	4	7.78	4.72
L. acidophilus BG2-FO4	0		8.90	8.85
	100	5	8.08	<1.70[a]
Lactobacillus GG ATCC 53103	0		9.34	9.22
	200	1	3.83	4.00

a Marginal growth

picked, but only 1,730 of these colonies grew on MRS agar and were replicaplated on 5% bMRS agar. The goal was to find colonies that would not grow on 5% bMRS agar.

After screening for bile tolerance, five colonies of strain ATCC 43121 were found with reduced bile tolerance. Isolate 26-1 did not grow on 5% bMRS agar, when the colony was initially transferred from MRS agar to 5% bMRS agar. Colonies II-35, 6-9, 6-10, and 7-13 grew on 2% bMRS agar, but did not grow on 5% bMRS agar (6-9, 7-13), or showed marginal growth (II-35, 6-10) on 5% bMRS agar after initial transfer from MRS agar.

A total of 300 colonies of strain ATCC 33198 was picked from MRS agar and tested on 2% and 5% bMRS agar. All of the colonies of ATCC 33198 grew on 2% and 5% bMRS except for two colonies. However, these two colonies were too injured to grow after transfer to MRS broth.

4.2.2 Comparison of the plasmid profiles of strain ATCC 43121 and the cured strains

The plasmid profiles of the selected isolates, 6-9, 6-10, 7-13, and 26-1, from the curing procedure were compared with the plasmid profile of the parental strain *L. acidophilus* ATCC 43121 to check for possible loss of plasmids. The results from the small scale plasmid isolation indicated that there were changes in the plasmid profiles of the ATCC 43121 cured strains 6-9, 6-10, 7-13, and 26-1 (Fig 2). The plasmid profiles of strains 6-9 and 7-13 were similar, both having three DNA bands at the same relative migration distance. Migration of the two lower bands of strains 6-9 and

7-13 differed from those of the wild type strain. Only the chromosomal DNA band was detected in the plasmid profiles of strains 6-10 and 26-1.

Based on the small scale plasmid isolation, it was difficult to assess whether the changes in the plasmid profile of strains 6-9, 6-10, 7-13, and 26-1 were due to loss of plasmids, rearrangements or problems with the procedure. A large scale plasmid isolation was done to produce larger amounts of DNA. For the large scale isolation, isolates 6-10 and 26-1 were chosen because of the apparent loss of two plasmids, and isolate 26-1 was also selected because of its original lack of growth on 5% bMRS agar after curing with novobiocin.

The plasmid profiles of variants 6-10 and 26-1 after large scale plasmid isolation (Figs 3 and 6) showed that the plasmid profiles of variants 6-10 and 26-1 had the same three DNA bands as the plasmid profiles of strains 6-9 and 7-13. *L. acidophilus* ATCC 43121 appeared to have two plasmids (Fig 3), approximately 2.8 and 5.4 MDa in size, as estimated based on the plasmids of the size marker *E. coli* V517 (Macrina et al., 1978) (Fig 4). The band underneath the chromosomal band of strain ATCC 43121 was likely open circular (OC) form of the 5.4 MDa plasmid (Fig 6). The band traveling between the 2.8 and 5.4 MDa plasmids of strain ATCC 43121 appeared to be the OC form of the 2.8 MDa plasmid, because both of the bands were lost in variants 6-10 and 26-1 (Fig 3 and 6). This was confirmed after purification of the DNA by cesium chloride-ethidium bromide density gradient centrifugation (Figs 5 and 7). The three DNA bands seen in the plasmid profile of the higher band from cesium chloride extraction of strain ATCC 43121 represented the chromosomal DNA band, and the OC forms of the 5.4 and 2.8 MDa plasmids. The 5.4 and 2.8 MDa plasmids were seen in the plasmid profile of the lower band from

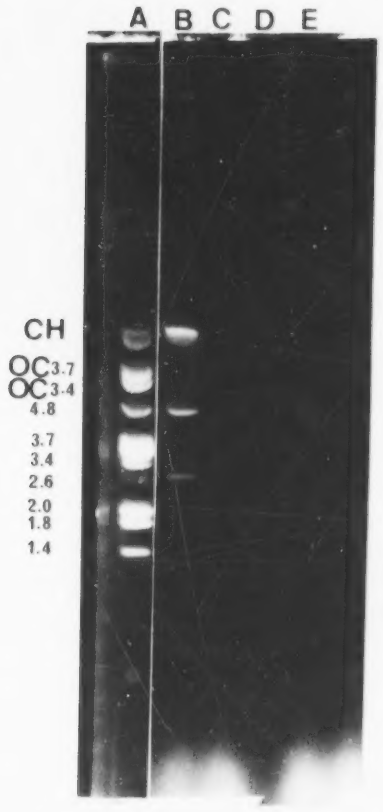

Fig 2. Plasmid profiles of *L. acidophilus* ATCC 43121 (B), and the cured variants 6-9 (C), 6-10 (D), 7-13 (E), and 26-1 (F), with plasmids of *Escherichia coli* V517 (A) as a size marker. CH indicates chromosomal DNA and OC indicates open circular DNA.

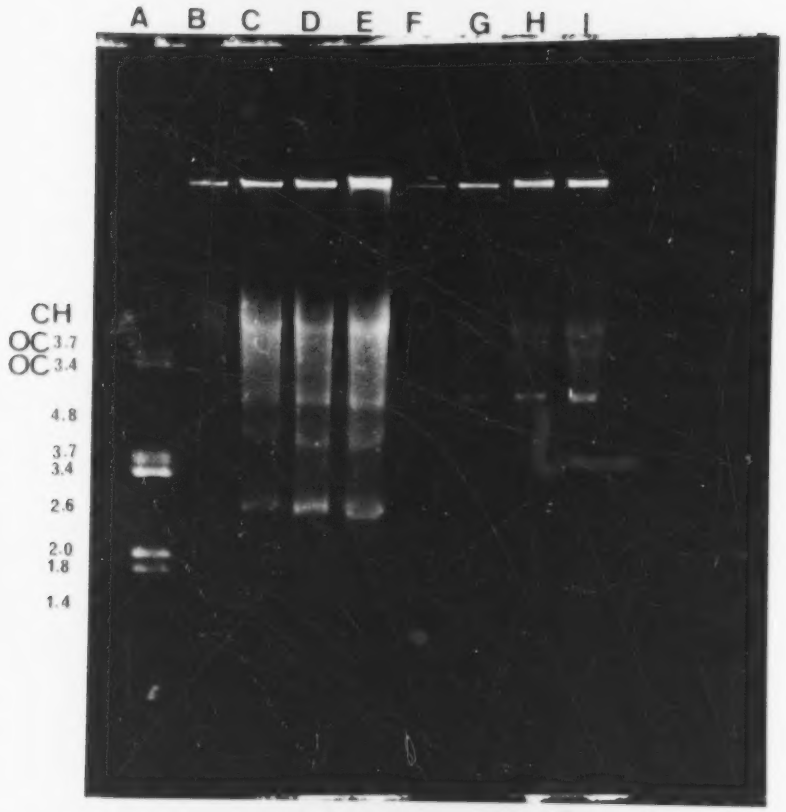

Fig 3. Plasmid profiles of *L. acidophilus* ATCC 43121 (5 µl, B; 10 µl, C; 15 µl, D; 20 µl, E) and variant 6-10 (5 µl, F; 10 µl, G; 15 µl, H; 20 µl, I) after large scale plasmid isolation, with plasmids of *Escherichia coli* V517 (A) as a size marker. Sizes of plasmids are expressed in MDa. CH indicates chromosomal DNA and OC indicates open circular DNA.

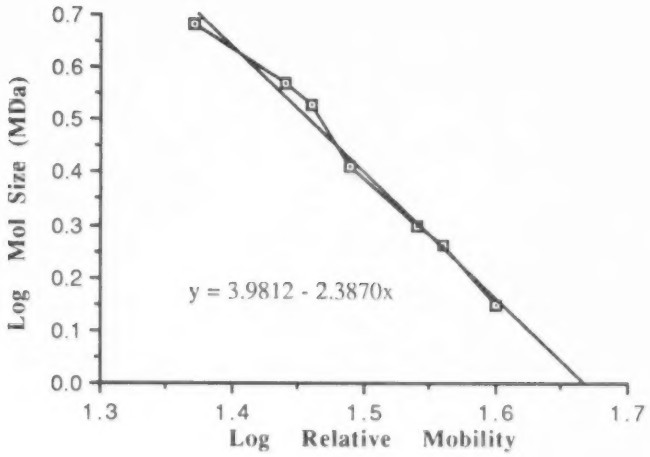

Fig 4. Migration of plasmids of *Escherichia coli* V517 relative to molecular size. Standard curve used for estimation of the size of the plasmids of *L. acidophilus* ATCC 43121.

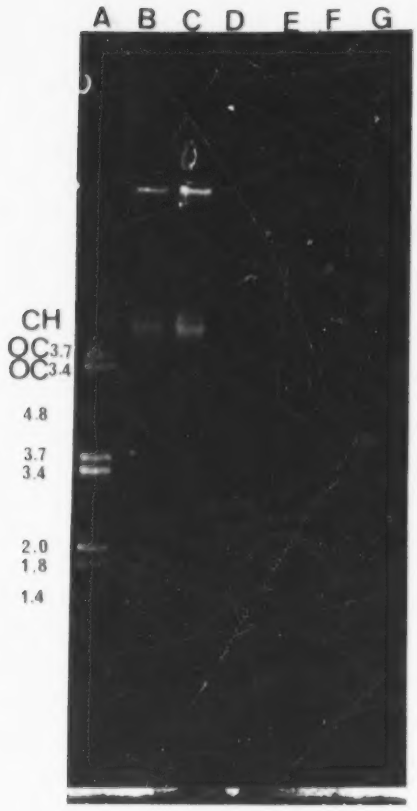

Fig 5. Plasmid profiles of *L. acidophilus* ATCC 43121 and variant 6-10 after purification on cesium chloride - ethidium bromide density gradient, with plasmids of *Escherichia coli* V517 as a size marker.

Lane: A. Size marker plasmids from *E. coli* V517; B-E. Upper band (B, 15 µl; C, 20 µl) and lower band (D, 15 µl; E 20 µl) of the cesium chloride extraction of *L. acidophilus* ATCC 43121; F-G. Cesium chloride extraction of variant 6-10 (F, 15 µl; G, 20 µl). CH indicates chromosomal DNA and OC indicates open circular DNA.

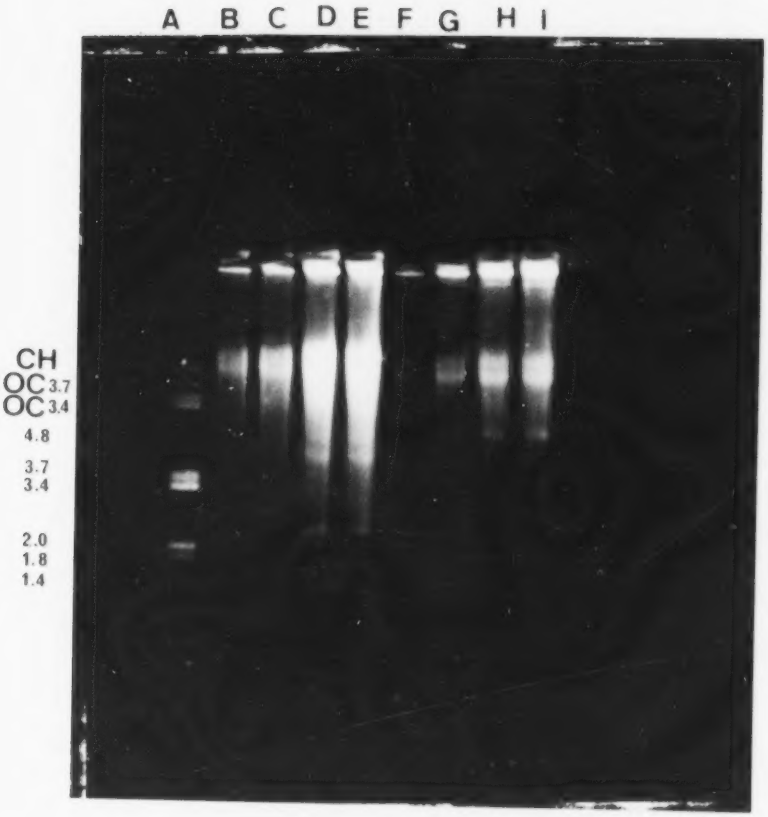

Fig 6. Plasmid profiles of *L. acidophilus* ATCC 43121 (5 µl, B; 10 µl, C; 15 µl, D; 20 µl, E) and variant 26-1 (5 µl, F; 10 µl, G; 15 µl, H; 20 µl, I) after large scale plasmid isolation, with plasmids of *Escherichia coli* V517 (A) as a size marker. Sizes of plasmids are expressed in MDa. CH indicates chromosomal DNA and OC indicates open circular DNA.

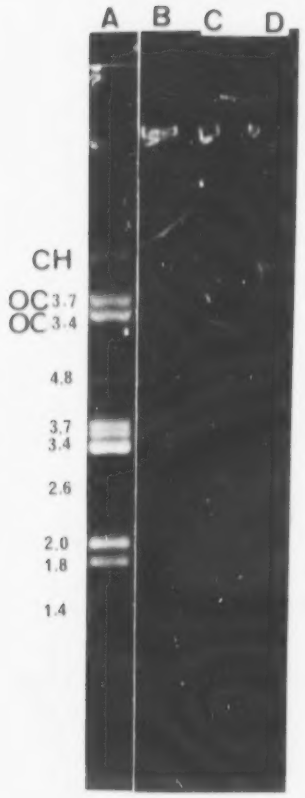

Fig 7. Plasmid profiles of *L acidophilus* ATCC 43121 and variant 26-1 after purification on cesium chloride - ethidium bromide density gradient, with plasmids of *Escherichia coli* V517 as a size marker.

Lane: A. Size marker plasmids from *E. coli* V517; B-C. Upper band (B) and lower band (C) of the cesium chloride extraction of *L. acidophilus* ATCC 43121;
D. Cesium chloride extraction of variant 26-1. CH indicates chromosomal DNA and OC indicates open circular DNA.

cesium chloride extraction of strain ATCC 43121. Variants 6-10 and 26-1 had lost the 2.8 MDa plasmid but the 5.4 MDa plasmid was not lost. The band between the chromosomal band and the band representing the 5.4 MDa plasmid in the plasmid profile of variant 6-10 was likely the open circular form of the 5.4 MDa plasmid (Fig 3). The chromosomal DNA band and the band representing the open circular form of the 5.4 MDa plasmid were seen in the plasmid profile of the cesium chloride extraction of variants 6-10 and 26-1 (Figs 5 and 7).

The 5.4 MDa plasmid appeared to be a low copy number plasmid, based on the plasmid profile of strain ATCC 43121 after the small scale plasmid extraction, in which the plasmid was seen as a weak band compared with the 2.8 MDa plasmid and its OC form in the same plasmid profile (Fig 2). Because it is a low copy number plasmid, the 5.4 MDa plasmid would show as a weak band in the tube after purification on cesium chloride - ethidium bromide density gradient, and was not detected when collecting the DNA bands from the cesium chloride tube.

Based on all of the plasmid profiles of strain ATCC 43121 and variants 6-9, 6-10, 7-13, and 26-1, it was concluded that the growth of *L. acidophilus* ATCC 43121 in MRS broth containing 13 µg/ml of novobiocin at 45°C resulted in curing of the 2.8 MDa plasmid but not the 5.4 MDa plasmid of variants 6-10 and 26-1.

4.2.3 Comparison of the bile tolerance of *L. acidophilus* ATCC 43121 and its cured variants

Strain ATCC 43121 and its cured variants II-35, 6-9, 6-10, 7-13, and 26-1 were tested for sensitivity to bile on MRS, and 2 and 5% bMRS agar. None of the strains was

Table 7. Counts of *L. acidophilus* ATCC 43121 and variants II-35, 6-9, 6-10, 7-13, and 26-1 on MRS and 2 and 5% bMRS agar after 48 h at 35°C. Counts are the mean of two determinations.

Strain	MRS agar (Log cfu/ml)	2% bMRS agar (Log cfu/ml)	5% bMRS agar (Log cfu/ml)
ATCC 43121	9.09	8.99	8.77
II-35	9.18	9.12	9.08
6-9	9.06	8.97	8.89
6-10	9.25	9.16	8.97
7-13	8.72	8.58	8.23
26-1	9.17	9.06	8.92

sensitive to bile (Table 7). All of the strains that had showed reduced growth or no growth on bMRS agar, were now able to grow on 5% bMRS agar in similar numbers as on MRS agar. By this time the strains had likely recovered from injury caused by the treatment with novobiocin.

L. acidophilus strain ATCC 43121 and the variant 26-1 were tested and compared for their viability in 0, 0.15, 0.3, 0.6, 1, 2, and 5% bMRS broth. The viability was determined by viable cell counts (cfu/ml) of these bMRS broth cultures on MRS agar. The results are presented in Fig 8, and the maximum population at each concentration of bMRS is listed in Table 8.

The 0.1% inocula of strains ATCC 43121 and 26-1 were calculated to be 6.11 and 6.17 log cfu/ml, respectively. During the 48 h incubation at 35°C, the viability of strains ATCC 43121 and 26-1 were similar in MRS and 0.15, 0.3, 2, and 5% bMRS broth. In 0.6 and 1% bMRS, strain 26-1 reached a higher population and remained in higher numbers than strain ATCC 43121. Both strains reached 8 log cfu/ml or higher population after up to 12 h incubation at 35°C in all the concentrations of bMRS. Both strains died off faster as the concentration of bile increased.

It can be concluded that no loss of bile tolerance of variants II-35, 6-9, 6-10, 7-13, and 26-1 could be interpreted from the tests for bile tolerance.

The use of a microplate reader to follow growth in the presence of bile was tested during the experiment on the viability of strains ATCC 43121 and 26-1 in bMRS containing different concentrations (0, 0.15, 0.3, 0.6, 1, 2, 5%) of bile. Absorbance (660 nm) of the samples, in duplicates was measured at the same time points as the sampling for the colony counts on MRS agar.

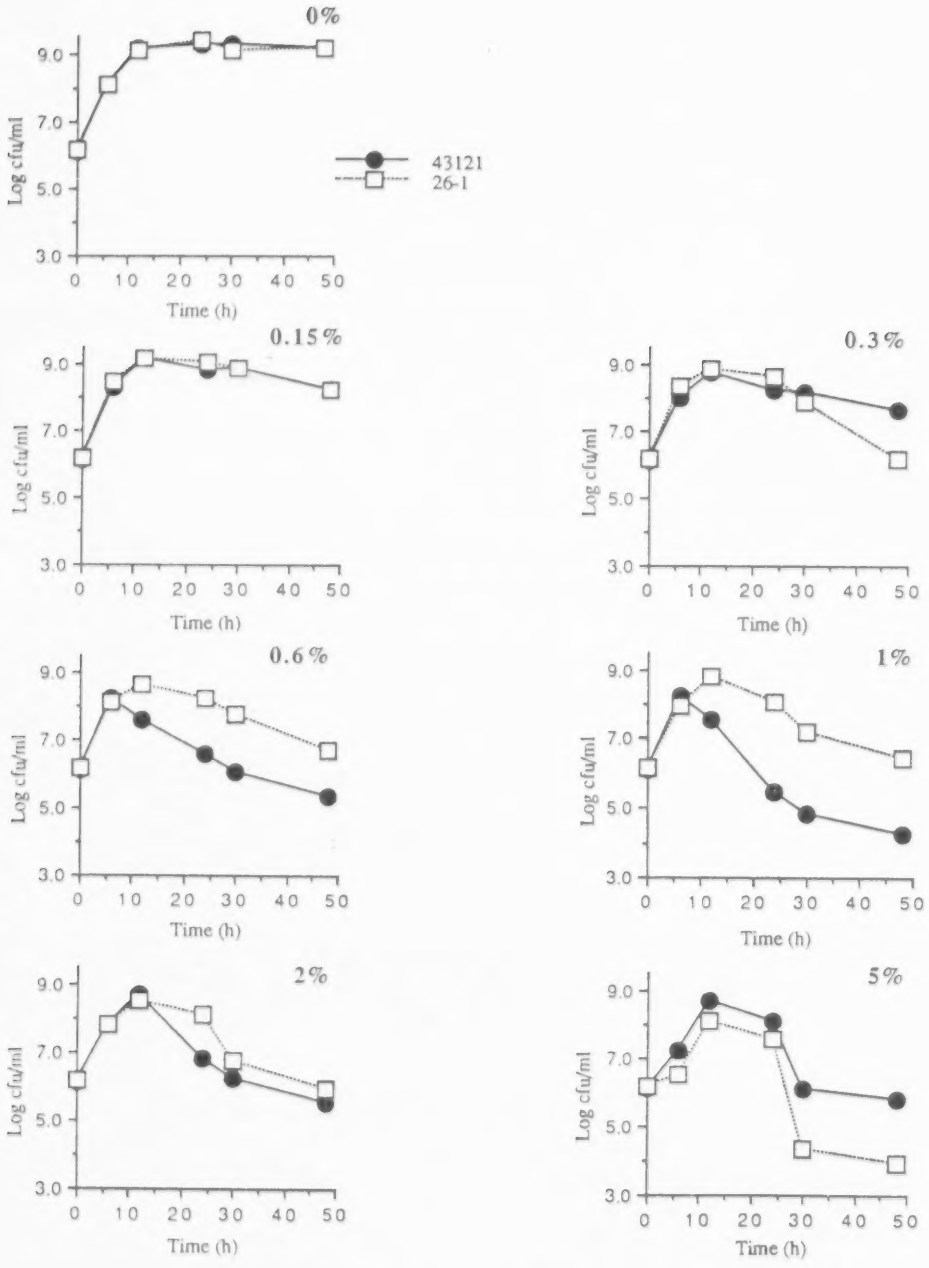

Fig 8. Comparison of the viability of *L. acidophlius* ATCC 43121 and variant 26-1 in 0, 0.15, 0.3, 0.6, 1, 2, and 5% bMRS.

Table 8. Maximum population of strains ATCC 43121 and 26-1 and hours of incubation at 35°C in various concentrations of bile. Results are based on one experiment.

Strain	% bMRS	Maximum population log cfu/ml	Time of incubation h
ATCC 43121	0	9.29	30
	0.15	9.13	12
	0.3	8.75	12
	0.6	8.23	6
	1	8.24	6
	2	8.69	12
	5	8.66	12
26-1	0	9.37	24
	0.15	9.17	12
	0.3	8.84	12
	0.6	8.61	12
	1	8.82	12
	2	8.48	12
	5	8.11	12

The absorbance data obtained with the microplate reader did not correlate with the viable cell counts (log cfu/ml), even when the differences in absorbance between various bile concentrations were taken into account using different blanks for different bile concentrations. For example, at 12 h the viable cell count of strain ATCC 43121 (9.13 log cfu/ml) in 0.15% bMRS was more than 1 log cfu/ml higher than the viable cell count in 1% bMRS (7.54 log cfu/ml; Fig 8), but the absorbance (660 nm) of the 0.15% bile sample (0.628) was considerably lower than the absorbance of the 1% bile sample (1.305). Also, as seen from the viability graphs (Fig 8), strain ATCC 43121 had died off to its lowest viable cell count in 5% bMRS (5.80 log cfu/ml) by 48 h, but the absorbance (660 nm) was at its highest (1.415) at that time point. Similar inconsistencies can be observed for strain 26-1.

4.2.4 Comparison of the fermentation profiles of *L. acidophilus* ATCC 43121 and the cured strains

The fermentation profiles of ATCC 43121 and the isolated strains from the curing procedure are presented in Table 9. The fermentation profiles of the isolates II-35, 6-9, 6-10, 7-13 and 26-1 were identical to that of the parental strain ATCC 43121.

4.3 INCREASED BILE TOLERANCE OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*

4.3.1 Selecting for increased bile tolerance

The ability of *L. bulgaricus* strains J9 and ATCC 11842 to increase their bile tolerance was studied by subculturing in increasing concentrations of bile. Both *L. bulgaricus* strains J9 and ATCC 11842 grew initially in 0.15% bMRS broth.

Table 9. The fermentation profiles of *L. acidophilus* ATCC 43121 and variants II-35, 6-9, 6-10, 7-13, and 26-1.

	ATCC 43121	II-35	6-9	6-10	7-13	26-1
amygdalin	+	+	+	+	+	+
cellobiose	+	+	+	+	+	+
fructose	+	+	+	+	+	+
galactose	+	+	+	+	+	+
gluconate	-	-	-	-	-	-
glucose	+	+	+	+	+	+
maltose	+	+	+	+	+	+
mannitol	-	-	-	-	-	-
mannose	+	+	+	+	+	+
ribose	-	-	-	-	-	-
salicin	+	+	+	+	+	+
sucrose	+	+	+	+	+	+
trehalose	+	+	+	+	+	+
xylose	-	-	-	-	-	-

\- negative

\+ positive

L. bulgaricus J9 and ATCC 11842 were repeatedly subcultured in increasing concentrations of bile in MRS broth, starting from 0.15% bMRS broth. Subculturing was repeated in the same concentration of bile in case growth did not occur at the increased concentration of bile. Eventually, strain J9 was able to grow in 1.6% bMRS. J9 isolates were obtained from different concentrations of bile as colonies of varying morphology. Two strains, J9-S and J9-V, were chosen for further study. J9-S was a smooth colony isolated from 1.2% bMRS broth, and J9-V was a rough colony isolated from 1.6% bMRS broth. In contrast, selection for increased bile tolerance of *L. bulgaricus* ATCC 11842 was not successful. This strain did not grow at bile concentrations above 0.15%.

The bile tolerance of strain J9 and variants J9-S and J9-V was compared by determining their population on bMRS agar and by following their viability in bMRS broth.

4.3.2 Comparison of the bile tolerance of strains J9, J9-S, and J9-V on bMRS agar

Colony counts of strains J9, J9-S, and J9-V on bMRS agar after 72 h of incubation at 35°C are shown in Table 10. Both variants J9-S and J9-V showed increased bile tolerance compared with strain J9. Growth of strain J9 was marginal above 0.3% bMRS agar, whereas growth of variants J9-S and J9-V was not affected by bile concentrations of up to 1% bMRS agar. On 2% bMRS agar, the counts of variants J9-S and J9-V were 6.26 and 7.01 log cfu/ml, respectively, which was two log cycles less than on MRS agar. Growth of variants J9-S and J9-V was marginal on 5% bMRS agar.

Table 10. The colony counts of J9, J9-S and J9-V on bMRS agar after 72 h of incubation at 35°C.

% bile in MRS agar	J9 (log cfu/ml)	J9-S (log cfu/ml)	J9-V (log cfu/ml)
0	8.26	8.45	8.92
0.15	6.78	8.51	8.72
0.3	< 3.70	8.43	8.74
0.6	< 3.70	8.29	8.75
1	< 2.70	7.76	8.86
2	< 2.70	6.26	7.01
5	< 2.70	<1.70	<2.70

4.3.3 Comparison of the viability of strains J9, J9-S, and J9-V in bMRS broth

Viability of strains J9, J9-S and J9-V was tested in 0, 0.15, 0.3, 0.6, 1, 2, 2.5, 3, 4, and 5% bMRS broth for 72 h at 35°C, and is presented in Figs 9 and 10. The maximum populations of strains J9, J9-S and J9-V at each concentration of bile are shown in Table 11. Results in Table 11 show that the maximum population of strains J9, J9-S, and J9-V were reduced as the concentration of bile in the medium was increased. From Figs 9 and 10 it can be seen that strain J9 was sensitive to bile and only grew at 0.15% bile in MRS broth, whereas strains J9-S and J9-V were bile tolerant and grew and remained viable for 72 h at bile concentrations of up to 4%. The growth curves of strain J9-S in 2, 2.5, 3, and 4%, and strain J9-V in 4% bMRS broth indicated a long lag-phase and eventual adaptation of the remaining viable cells to the high concentration of bile. None of the strains grew in 5% bMRS broth.

4.3.4 Comparison of *L. delbrueckii* subsp. *bulgaricus* strain J9 and variants J9-S and J9-V by fermentation profiles and coagulation of skim milk

The carbohydrate fermentation profiles and ability to coagulate skim milk of strains J9-S and J9-V was tested and compared with strain J9. The fermentation profiles of 17 sources of energy of strains J9, J9-S and J9-V were determined on carbohydrate fermentation agar to check for possible changes in the profiles of variants J9-S and J9-V. The fermentation profiles are presented in Table 12. There were no differences in the fermentation profiles of variants J9-S and J9-V compared with strain J9. Strains J9, J9-S, and J9-V coagulated skim milk after 24 h incubation at 35°C.

Table 11. Maximum population of *L. delbrueckii* subsp. *bulgaricus* strains J9, J9-S and J9-V and time of incubation at 35°C at different concentrations of bile.

Strain	% bMRS	Maximum population log cfu/ml	Time of incubation h
J9	0	8.47	24
	0.15	6.25	72
	0.3	5.08	24
	0.6	5.02	24
	1	5.06	8
	2	4.39	8
	2.5	3.9	8
	3	3.75	8
	4	3.41	8
	5	3.23	8
J9-S	0	8.48	48
	0.15	8.09	48
	0.3	7.87	48
	0.6	7.64	72
	1	6.97	72
	2	6.07	72
	2.5	5.53	72
	3	5.27	72
	4	4.64	8
	5	4.04	8
J9-V	0	8.85	24
	0.15	7.69	48
	0.3	8.05	24
	0.6	7.65	24
	1	7.62	48
	2	7.27	48
	2.5	6.87	72
	3	7.07	48
	4	5.45	72
	5	4.54	8

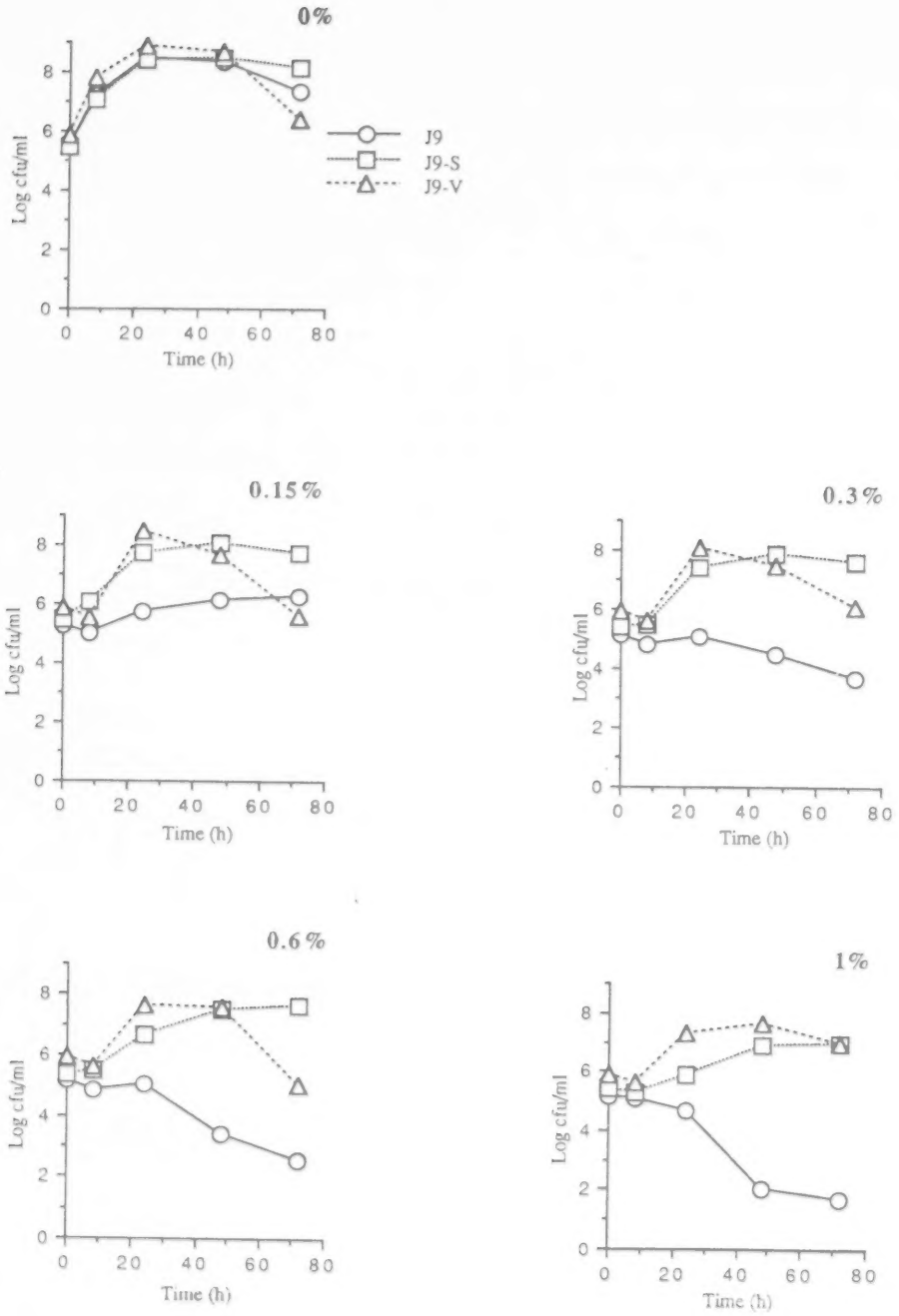

Fig 9. Comparison of viability of *L. delbrueckii* subsp. *bulgaricus* strains J9, J9-S, and J9-V in 0, 0.15, 0.3, 0.6, and 1% bMRS.

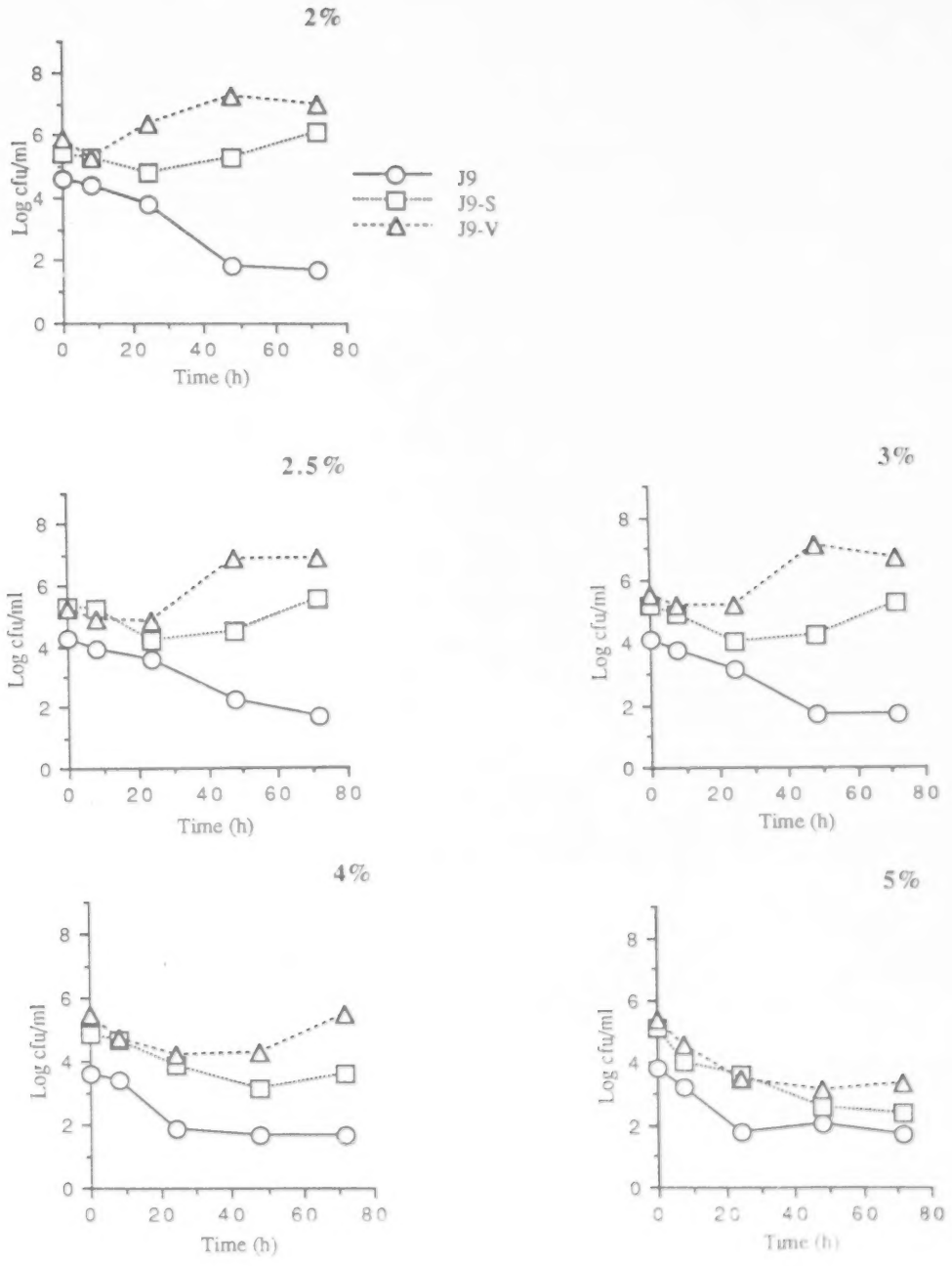

Fig 10. Comparison of viability of *L. delbrueckii* subsp. *bulgaricus* strains J9, J9-S, and J9-V in 2, 2.5, 3, 4, and 5% bMRS.

Table 12. Comparison of the fermentation profiles of strains J9, J9-S , and J9-V on carbohydrate fermentation agar.

	J9	J9-S	J9-V
arabinose	-	-	-
amygdalin	-	-	-
cellobiose	-	-	-
fructose	+	+	+
galactose	-	-	-
gluconate	-	-	-
glucose	+	+	+
lactose	+	+	+
maltose	-	-	-
mannitol	-	-	-
mannose	+	+	+
raffinose	-	-	-
ribose	-	-	-
salicin	-	-	-
sucrose	-	-	-
trehalose	-	-	-
xylose	-	-	-

\+ positive

\- negative

5. DISCUSSION AND CONCLUSIONS

Differences in bile tolerance within *Lactobacillus* sp. have been observed (Gilliland and Speck, 1977b). The non-intestinal *L. delbrueckii* subsp. *bulgaricus* has been reported to be more sensitive to bile than the intestinal organism *L. acidophilus*. Gilliland et al. (1984) observed considerable variation in the bile tolerance of seven strains of *L. acidophilus* isolated from the intestines of calves. Because of this variation in bile tolerance within *Lactobacillus* spp., the possibility of bile tolerance being plasmid-mediated was studied. Loss of a plasmid together with the loss of bile tolerance would indicate the strong possibility that bile tolerance is plasmid-mediated. This was of interest given the possibility of developing a probiotic strain that would be competitive in the intestinal environment and possibly colonize the intestinal tract.

Twelve strains of *Lactobacillus* spp. were screened for their bile tolerance. Five strains of *L. acidophilus*, six strains of *L. delbrueckii* subsp. *bulgaricus*, and a strain of *Lactobacillus* GG were chosen for initial bile tolerance testing. The three *Lactobacillus* species were chosen for testing because of their particular characteristics. The traditional yoghurt starter organism *L. delbrueckii* subsp. *bulgaricus* was reported to be sensitive to bile while the intestinal organism *L. acidophilus* was reported to be resistant to bile (Gilliland and Speck, 1977b). *Lactobacillus* GG, a widely researched human probiotic organism, was originally selected with its tolerance for bile (0.15%) among the selection criteria (Goldin et al., 1992; Silva et al., 1987). The object of screening in this study was to find a highly bile tolerant *Lactobacillus* strain and a bile sensitive *Lactobacillus* strain, which could be used in experiments on bile tolerance. Screening was done by testing for bile tolerance in MRS broth and on MRS agar containing different concentrations of bile. Based on the results, *L. acidophilus* strains ATCC 43121, ATCC 33198, and

BG2FO4, *Lactobacillus* GG strain ATCC 53103, and *L. delbrueckii* subsp. *bulgaricus* strains ATCC 11842 and J9 were selected for further experiments on their bile tolerance.

L. acidophilus strains ATCC 33198, ATCC 43121 and BG2-FO4, and *Lactobacillus* GG strain ATCC 53103, which all grew in 10% bMRS broth and on 10% bMRS agar, were chosen for plasmid curing by growing them in the presence of novobiocin at 45°C. Loss of bile tolerance was monitored by differential counting on MRS agar and 5% bMRS agar. The results of the differential counting of strain ATCC 53103 did not indicate loss of bile tolerance as the growth of strain ATCC 53103 was similar both on MRS agar and 5% bMRS agar. No growth of strains ATCC 33198 or BG2-FO4 was observed on 5% bMRS agar after the curing procedure, which indicated injury of the two strains due to the curing process. A total of 300 colonies of strain ATCC 33198 were screened for their bile tolerance on 5% bMRS agar. The two colonies of strain ATCC 33198 that did not grow on 5% bMRS agar, during screening, appeared to be injured because they also did not grow in MRS broth.

The differential counts of strain ATCC 43121 on MRS and 5% bMRS agar indicated a 1:1,000 chance of finding a mutant which had lost its bile tolerance. Because of this, 1,730 colonies of strain ATCC 43121 were picked and screened for their bile tolerance on 5% bMRS agar. During the screening, ATCC 43121 variants II-35, 6-9, 6-10, 7-13, and 26-1 did not grow or had reduced growth on 5% bMRS agar, however, this was shown to be due to injury. After recovery in MRS broth, all five of the ATCC 43121 variants grew in similar numbers on both MRS agar and 5% bMRS agar. The viability of variant 26-1 and its parental strain ATCC 43121 was compared in 0.15, 0.3, 0.6, 1, 2, and 5% bMRS, but no significant difference was found between these

strains. In this study, no *Lactobacillus* strains were found which had lost their bile tolerance.

Strain ATCC 43121 was shown to have two plasmids, which were estimated to be 5.4 and 2.8 MDa in size, based on the plasmids of *Escherichia coli* V517 size marker (Macrina et al., 1978). As a result of the curing procedure, ATCC 43121 variants 26-1 and 6-10 were cured of the 2.8 MDa plasmid. Since no loss of bile tolerance was observed in the cured strains 26-1 and 6-10, it was concluded that the 2.8 MDa plasmid of strain ATCC 43121 did not mediate bile tolerance. Consequently, this study did not indicate that bile tolerance would be plasmid-mediated, but only the curing of the 5.4 MDa plasmid, and the effect of its loss on the bile tolerance of strain ATCC 43121 would fully answer the question whether the bile tolerance of strain ATCC 43121 is plasmid-mediated.

In the course of the study, injured colonies of *L. acidophilus* strains ATCC 43121, ATCC 33198, and BG2-FO4 were observed to be more sensitive to bile than non-injured colonies. Increased sensitivity of *L. acidophilus* to bile has been reported due to injury from freezing, freeze drying and vacuum drying (Johnson et al., 1984; Brennan et al., 1986). In addition, Kole and Altosaar (1984) reported that a bile resistant *Leuconostoc oenos* strain survived lyophilization better than bile sensitive strains. These observations suggest that the physiological state of the bacterial cell is important in determining its survival in bile.

In the intestinal environment, probiotic organisms may be injured by the acidity of the stomach when entering the duodenum, where they are also exposed to bile. *L. acidophilus* has been reported to survive in human gastric juice in vitro as well as or better than *L. delbrueckii* subsp. *bulgaricus*, which survived very poorly in human

gastric juice in vivo (Conway et al., 1987; Robins-Browne and Levine, 1981). The products of interest in this study, namely yogurt and other dairy products, may have a protective effect against the acidity of the stomach (Conway et al., 1987). According to studies done by M. Bennik in our laboratory in 1992, the exposure of 15 strains of *Lactobacillus* sp. (10% inoculum) to pH 2.5 for 6 hours did not affect the bile tolerance of the strains. However, pH 2.0 was lethal for most of these 15 lactobacilli, which included 6 strains of *L. acidophilus*, 2 strains of *L. bulgaricus*, 3 strains of *L. casei* (including *Lactobacillus* GG), and 4 intestinal *Lactobacillus* isolates. Strains ATCC 43121 and J9 were not among the strains tested. The final pH of the cultures of these 15 strains of *Lactobacillus* sp. in MRS varied from pH 3.80 to pH 3.90.

There are some reports of increased bile tolerance of bacteria after exposure to bile. The bile tolerance of a strain of *Leuconostoc oenos* was improved by exposing the strain to increasing concentrations of bile (Kole and Altosaar, 1984). Some oral bacteria (*Bacteroides*, *Bifidobacterium*, and *Veillonella* spp.) which were sensitive to bile, survived repeated subculturing in 10% bile (Hill and Drasar, 1972). These results suggest that bile tolerance is at least partly a physiological phenomenon.

In this study, the ability of bile sensitive *L. delbrueckii* subsp. *bulgaricus* to adapt to bile was determined. This was done by exposing *L. delbrueckii* subsp. *bulgaricus* strains J9 and ATCC 11842 to increasing concentrations of bile in order to enhance their viability in the presence of bile. Strains J9 and ATCC 11842 were observed to be sensitive to bile in the initial screening of the strains of *Lactobacillus* spp. As a result of repeated subculturing in the presence of bile, the bile tolerance of *L. delbrueckii* subsp. *bulgaricus* J9 was increased, and two variants J9-S and J9-V, with increased viability in bile, were obtained. Growth of strain J9 was reduced on 0.15% bMRS agar and marginal on 0.3, 0.6, 1, and 2% bMRS agar, whereas growth of

variants J9-S and J9-V was only affected by 2% bile in MRS agar. The increased tolerance of strains J9-S and J9-V to bile remained stable during subculturing in MRS (without bile). Whether prolonged subculturing in MRS (without bile) would result in the loss of increased viability in bile would be useful to know but was not determined in this study. Increased bile tolerance may be due to changes in the lipid content of the cell membrane. Kole and Altosaar (1984) reported changes in the membrane lipid profile of *Leuconostoc oenos* after it had been subcultured and adapted in increasing concentrations of bile. Although time did not permit in this study, it would be interesting to analyze and compare the lipid content of the membrane of strains J9, J9-S, and J9-V for possible changes after the selection for increased bile tolerance.

Different bile acids have different inhibitory activities. Deconjugated bile acids have been found to be more inhibitory to bacteria. About 95% of the bile acids in freshly excreted bile in the duodenum are conjugated and thus antibacterially less potent than the free bile acids resulting from the microbial deconjugation that occurs in the distal intestine (Sung et al., 1993). The significance of bile deconjugation to lactobacilli and other bacteria has not been fully clarified (Floch et al., 1972; Gilliland and Speck, 1977a; Tannock et al., 1989). Because free bile acids are more inhibitory than conjugated bile acids, deconjugation may have a role in microbial competition in the intestines. The chromosomal gene encoding the ability to deconjugate bile (i.e. the production of bile hydrolase enzyme) has been located in lactobacilli (Christiaens et al., 1992), but the involvement of plasmids has not been reported. The ability to deconjugate bile may have an effect on bile tolerance of a strain. Klaver and van der Meer (1993) reported that the *L. acidophilus*, *L. casei* , and *Bifidobacterium bifidum* strains that deconjugated bile salts showed low biomass concentrations after incubation in the presence of oxgall compared with the amount of biomass obtained in MRS medium without oxgall. The explanation may lie in the inhibitory action of the

released unconjugated bile acids on the organism itself. Some oral bacteria (*Bacteroides*, *Bifidobacterium*, and *Veillonella*) have been found to acquire the ability to deconjugate bile after being exposed several times to 10% bile (Hill and Drasar, 1968).

The repeated exposure of strain J9 to bile could have had an effect on its bile deconjugation activity, as was observed by Drasar and Hill (1968). Whether or not strain J9 and variants J9-S and J9-V deconjugate bile, is not known. It would be useful to compare information on the bile acid composition and the pHs of the media, both before and after the experiment, and the pKas of the bile acids in question.

The attempt to use a microplate reader for comparing the growth of ATCC 43121 and isolate 26-1 in bMRS containing various concentrations (0, 0.15, 0.3, 0.6, 1, 2, and 5%) of bile, was not successful. The absorbance curves (not presented) obtained with the microplate reader (660 nm) did not correlate with the viability curves obtained by plating the bMRS cultures on MRS agar at the same time points. Besides the effect of dead cells, the high absorbances obtained when the viable cell counts (log cfu/ml) were low, were likely due to the deconjugation and precipitation of bile acids, which cause turbidity. Deconjugation releases bile acids that have a higher pKa than in their conjugated form and are thus precipitated more readily, which could be mistaken as a sign of growth. ATCC 43121 deconjugates bile (Gilliland et al., 1985). From previous work done in the Food Microbiology laboratory by M. Bennik in 1992, it is known that addition of up to 10% bile to MRS medium does not affect the pH of the medium before or after autoclaving. The final growth pH of ATCC 43121 in MRS broth was pH 3.90, and the pKas of cholic and deoxycholic acid are 6.4 and 6.58, respectively (The Merck Index, 1983). Floch et al. (1972) reported that *Bacteroides fragilis* and *Bacteroides oralis* cultures became extremely cloudy when grown in the

presence of bile but there were very few viable organisms within the tubes. According to Floch et al. (1972), the cloudiness appeared to be caused by precipitation of bile salts.

Due to bile deconjugation activity of bacteria and due to the inability to differentiate between live and dead cells, the method of observing turbidity either visually or spectrophotometrically as a sign of growth in bMRS tubes may not be a reliable measurement of viability. However, spectrophotometric measurements for growth in bile have been used in the literature (Gilliland et al., 1985; Noh and Gilliland, 1993). In those experiments, growth was observed for relatively short periods of time (e.g. for 8 hours), in which case spectrophotometric measurement may be appropriate. Turbidity measurements might be used for comparison within the same bile concentration. Even when blanks of appropriate bile concentration were used, there were differences due to the different bile concentrations. This was likely due to the different degrees of bile conjugation and thus precipitation that was taking place in the varying concentrations of bile.

The six species (*L. acidophilus*, *L. crispatus*, *L. gasseri*, *L. gallinarum*, *L. johnsonii*, and *L. amylovorus*) of the *L. acidophilus* group cannot be distinguished using the traditional physiological/phenotypical tests (Cato et al., 1983; Johnson et al., 1980; Lauer and Kandler, 1980). The 16S RNA profiles required for full identification were not used in this study. For the purpose of this experiment, because we were looking for changes in the fermentation patterns of the strains tested, more than their identification, the fermentation patterns obtained with API 50 CHL test strips and testing on carbohydrate fermentation agar, together with the results obtained from testing for the coagulation of skim milk, were sufficient. There were no differences in the fermentation profiles of the wild type strains *L. acidophilus* ATCC 43121 and *L.*

delbrueckii subsp. *bulgaricus* J9, and their variants II-35, 6-9, 6-10, 7-13 and 26-1, and J9-S and J9-V, respectively.

L. acidophilus ATCC 43121 fermented lactose, but did not coagulate skim milk in 24 hours. The reason may be that although *L. acidophilus* ferments lactose, it is known to grow slowly in milk (Mital and Garg, 1992). Both *L. bulgaricus* strain J9 and its variants J9-S and J9-V coagulated skim milk in 24 hours, as would be expected since *L. bulgaricus* grows well in milk and is used in yoghurt manufacture (Gilliland, 1985).

As was discussed in the literature review of this thesis, bile tolerance is among the properties required for an effective probiotic microorganism. A probiotic organism should at least survive the physiological bile concentrations, but to be competitive against other microorganisms in the intestine, a probiotic organism should be able to grow in the presence of bile. Because *L delbrueckii* subsp. *bulgaricus* variants J9-S and J9-V were found to be more bile tolerant than strain J9, they should have better competitive edge in the gastrointestinal tract. This study would have provided great potential for further study if the bile tolerance was plasmid-mediated in *L. acidophilus* ATCC 43121. Although bile tolerance was not proven to be plasmid-mediated in this study, the possibility remains and raises some interesting questions. Considering that bile is hypothesized to be an important the defense mechanism of the human body, the possibility that a pathogen could acquire a bile tolerance, via a plasmid, might lead to the break down of defense mechanisms in the human body. Considering the rare but yet occurring cases of involvement of *Lactobacillus* spp. in human clinical infections (Aguirre and Collins, 1993; Gasser, 1994), great care should be taken in selecting a probiotic organism. Because the intestinal environment is a very complex ecosystem, which is difficult to study in vitro, the simulated intestinal models reported by some

researchers (Marteau et al., 1993; Minekus et al., 1993; Molly et al., 1993; Savoie and Gauthier, 1986) are likely to be useful in this field of research.

6. REFERENCES

Aguirre, M., and M.D. Collins. 1993. Lactic acid bacteria and human clinical infection. J. Appl. Bacteriol. 75: 95-107.

Akiyoshi, T., and F. Nakayama. 1990. Bile acid composition in brown pigment stones. Dig. Dis. Sci. 35: 27-32.

Anderson, D.G., and L.L. McKay. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. 46: 549-552.

Anonymous. 1995. 'Prebiotics' in functional foods improve gut microflora: U.K. study. World Food Chemical News 1(46): 9.

Bibel, D.J. 1988. Elie Metchnikoff's bacillus of long life. ASM News 54: 661-665.

Borgström, B., J.A. Barrowman, and M. Lindström. 1985. Roles of bile acids in intestinal lipid digestion and absorption, p. 405-425. In Sterols and bile acids. H. Danielsson and J. Sjövall (ed.). Elsevier Science Publishers B. V. (Biomedical Division), The Netherlands.

Bottazzi, V. 1983. Other fermented dairy products, p. 315-363. In Biotechnology: Vol. 5 Food and Feed Production with Microorganisms (G. Reed, ed.) Verlag Chemie, Germany.

Bowden, T.A., A.R. Mansberger, and L.E. Lykins. 1981. Pseudomembraneous enterocolitis: mechanism of restoring floral homeostasis. Am. Surg. 47: 178-183.

Brennan, M., B. Wanismail, M. C. Johnson, and B. Ray. 1986. Cellular damage in dried *Lactobacillus acidophilus*. J. Food Prot. 49: 47-53.

Cato, E.P., W.E.C. Moore, and J.L. Johnson. 1983. Synonymy of strains of "*Lactobacillus aicdophilus*" group A2 (Johnson et al. 1980) with the type strain of *Lactobacillus crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970. Int. J. Syst. Bacteriol. 33: 426-428.

Chipman, J.K. 1982. Bile as a source of potential reactive metabolites. Toxicology 25: 99-111.

Christiaens, H., R.J. Leer, P.H. Pouwels, and W. Verstraete. 1992. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. Appl. Environ. Microbiol. 58: 3792-3798.

Collins, F.M., and P.B. Carter. 1978. Growth of Salmonellae in orally infected germfree mice. Infec. Immun. 21: 41-47.

Collins, M.D., B.A. Phillips, and P. Zanoni. 1989. Deoxyrobonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov. Int. J. Syst. Bacteriol. 39:105-108.

Collins, M.D., U. Rodrigues, C. Ash, M. Aguirre, J.A.E. Farrow, A. Martinez-Murcia, B.A. Phillips, A.M. Williams, and S. Wallbanks. 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol. Lett. 77: 5-12.

Collins, M.D., J. Samelis, J. Metaxopoulos, and S. Wallbanks. 1993. Taxonomic studies on some leuconostoc-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. J. Appl. Bacteriol. 75: 595-603.

Conway, P.L., S.L. Gorbach, and B.R. Goldin. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. J. Dairy Sci. 70: 1-12.

Daly, C. 1991. Lactic acid bacteria and milk fermentations, p. 544-548. In Biotechnology group meeting. Probiotics - fact or fiction? J. Chem. Tech. Biotechnol. 51: 539-570.

Dashkevicz, M.P., and S.D. Feighner. 1989. Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. Appl. Environ. Microbiol. 55: 11-16.

De Smet, I., L. Van Hoorde, N. De Saeyer, M. Vande Woestyne, and W. Werstraete. 1994. *In vitro* study of bile salt hydrolase (BSH) activity of BSH isogenic *Lactobacillus plantarum* 80 strains and estimation of cholesterol lowering through enhanced BSH activity. Microbial Ecology in Health and Disease 7: 315-329.

Dellaglio, F., L.M.T. Dicks, M. duToit, and S. Torriani. 1991. Designation of ATCC 334 in place of ATCC 393 (NCDO 161) as the neotype strain of *Lactobacillus casei* subsp. *casei* and rejection of the name *Lactobacillus paracasei* (Collins et al., 1989). Request for an opinion. Int. J. Syst. Bacteriol. 41: 340-342.

Difco. 1984. Difco Manual, tenth edition, Difco Laboratories Incorporated, Detroit Michigan 48232 U.S.A.

Dowling, R.H. 1972. The enterohepatic circulation. Gastroenterology 62: 122-140.

Drasar, B.S., and M.J. Hill. 1974. Human intestinal flora. Academic Press, London, Great Britain. pp. 103-123.

Feighner, S.D., and M.P. Dashkevicz. 1987. Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial choloyltaurine hydrolase activity. Appl. Environ. Microbiol. 53: 331-336.

Feighner, S.D., and M.P. Dashkevicz. 1988. Effect of dietary carbohydrates on bacterial hydrolase in poultry intestinal homogenates. Appl. Environ. Microbiol. 54: 337-342.

Finegold, S.M., V.L. Sutter, and G.E. Mathisen. 1983. Normal indigenous intestinal flora, p. 3-31. In Human intestinal microflora in health and disease. Hentges, D.J. (ed.). Academic Press, New York, U.S.A.

Finegold, S.M., V.L. Sutter, P.T. Sugihara, H.A. Elder, S.M. Lehmann, and R.L. Phillips. 1977. Fecal microbial flora in Seventh Day Adventist populations and control subjects. Am. J. Clin. Nutr. 30: 1781-1792.

Floch, M.H., H.J. Binder, B. Filburn, and W. Gershengoren. 1972. The effect of bile acids on intestinal microflora. Am. J. Clin. Nutr. 25: 1418-1426.

Floch, M.H., W. Gershengoren, S. Diamond, and T. Hersh. 1970. Cholic acid inhibition of intestinal bacteria. Am. J. Clin. Nutr. 23: 8-10.

Fox, S. I. 1984. Human physiology. Wm. C. Brown Publishers, Dubuque, Iowa, U.S.A.

Fujisawa, T., Y. Benno, T. Yaeshima, and T. Mitsuoka. 1992. Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of the *L. gallinarum* sp. nov. and *L. johnsonii* sp. nov. and synonymy of *L. acidophilus* group A3 (Johnson et al. 1980) with the type strain of *L. amylovorus* (Nakamura 1981). Int. J. Syst. Bacteriol. 42: 487-491.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66: 365-378.

Fuller, R. 1991. Probiotics in human medicine. Gut 32: 439-442.

Fuller, R. 1992. History and development of probiotics, p.1-8. In Probiotics the scientific basis. R. Fuller (ed.). Chapman & Hall, London, Great Britain.

Gasser, F. 1994. Safety of lactic acid bacteria and their occurrence in human clinical infections. Bull. Inst. Pasteur 92: 45-67.

Gilliland, S.E. 1985. The lactobacilli: milk products, pp.41-55. In Bacterial starter cultures for foods. S.E. Gilliland (ed.) CRC Press, Inc., Boca Raton, Florida, U.S.A.

Gilliland, S.E., C.R. Nelson, and C. Maxwell. 1985. Assimilation of cholesterol by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 49: 377-381.

Gilliland, S.E., and M.L. Speck. 1977a. Deconjugation of bile acids by intestinal lactobacilli. Appl. Environ. Microbiol. 33: 15-18.

Gilliland, S.E, and M.L. Speck. 1977b. Enumeration and identity of lactobacilli in dietary products. J. Food Prot. 40: 760-762.

Gilliland, S.E., T.E. Staley, and L.J. Bush. 1984. Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. J. Dairy Sci. 67: 3045-3051.

Goldin, B.R., and S.L. Gorbach. 1992. Probiotics for humans, p. 355-375. In Probiotics the scientific basis. R. Fuller (ed.). Chapman & Hall, London, UK.

Goldin, B.R., S.L. Gorbach, M. Saxelin, S. Barakat, L. Gualtieri, and S. Salminen. 1992. Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. Digestive Diseases and Sciences 37: 121.128.

Gorbach, S.L., T.-W. Chang, and B. Goldin. 1987. Succesful treatment of relapsing *Clostridium difficile* colitis with *Lactobacillus* GG. Lancet 2: 1519.

Goulet, J., and C. Matar. 1993. Production of bioactive peptides, p. 16-21. In Lactic acid bacteria research in Canada. Proceedings of Workshop, University of Alberta, Edmonton.

Gracey, M. 1983. The contaminated small bowel syndrome, p. 495-515. In Human intestinal microflora in health and disease. Hentges, D.J. (ed.). Academic Press, New York, U.S.A.

Havenaar, R., B. Ten Brink, and J.H.J. Huis in't Veld. 1992. Selection of strains for probiotic use, p.209-224. In Probiotics the scientific basis. R. Fuller (ed.). Chapman & Hall, London, UK.

Heimdahl, A., and C.E. Nord. 1979. Effect of phenoxymethylpenicillin and clindamycin on the oral, throat and faecal microflora of man. Scand. J. Infect. Dis. 11: 233-242.

Herter, C.A., and A.I. Kendall. 1908. An observation on the fate of *B. bulgaricus* (in Bacillac) in the digestive tract of a monkey. J. Biol. Chem. 5: 293-302.

Hill, M.J. 1990. Factors controlling the microflora of the healthy upper gastrointestinal tract, p. 57-85. In Human microbial ecology. M.J. Hill, and P.D. Marsh (ed.). CRC Press, Inc., Boca Raton, FLA, U.S.A.

Hill, M.J., and B.S. Drasar. 1968. Degradation of bile salts by human intestinal bacteria. Gut 9: 22-27.

Hill, M.J. , and P.D. Marsh (ed.). 1990. Human microbial ecology. CRC Press, Inc., Boca Raton, FLA, U.S.A.

Hose, H., and T. Sozzi. 1991. Probiotics, fact or fiction, p. 540-544. In Biotechnology group meeting. Probiotics - fact or fiction? J. Chem. Tech. Biotechnol. 51: 539-570.

Hughes, D.B., and D.G. Hoover. 1991. Bifidobacteria: Their potential for use in American dairy products. Food Technol. 45(4): 74-83.

Huis in't Veld, J.H.J., and R. Havenaar. 1991. Probiotics and health in man and animal, p. 562-567. In Biotechnology group meeting. Probiotics - fact or fiction? J. Chem. Tech. Biotechnol. 51: 539-570.

Hylemon, P.B., and T. Glass. 1983. Biotransformation of bile acids and cholesterol by the intestinal microflora, p.189-213. In Human intestinal microflora in health and disease. Hentges, D.J. (ed.). Academic Press, New York, U.S.A.

Isolauri, E., M. Juntunen, T. Rautanen, P. Sillanaukee, and T. Koivula. 1991. A human *Lactobacillus* strain (*Lactobacillus casei* sp. strain GG) promotes recovery from acute diarrhea in children. Pediatrics 88: 90-97.

Johansson, M.-L., G. Molin, B. Jeppson, S. Nobaek, S. Ahrné, and S. Bengmark. 1993. Administration of different *Lactobacillus* strains in fermented oatmeal soup: In vivo colonization of human intestinal mucosa and effect on the indigenous flora. Appl. Environ. Microbiol. 59: 15-20.

Johnson, J.L., C.F. Phelps, C.S. Cummins, J. London, and F. Gasser. 1980. Taxonomy of the *Lactobacillus acidophilus* group. Int. J. Syst. Bacteriol. 30: 53-68.

Johnson, M.C., B. Ray, and T. Bhowmik. 1987. Selection of *Lactobacillus acidophilus* strains for use in "acidophilus products". Antonie van Leeuwenhoek 53: 215-231.

Johnson, M., B. Ray, and M.L. Speck. 1984. Freeze-injury in cell wall and its repair in *Lactobacillus acidophilus*. Cryo-Letters 5: 171-176.

Kandler, O., and N. Weiss. 1986. Genus *Lactobacillus*, p. 1208-1234. In Bergey's Manual of Systematic Bacteriology Vol. 2. P. H. A. Sneath, N. Mair, and M.E. Sharpe, (Eds.). Williams and Wilkins, Baltimore, MD.

Kaila, M., E. Isolauri, E. Soppi, E. Virtanen, S. Laine, and H. Arvilommi. 1992. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human *Lactobacillus* strain. Pediatric Research 32: 141-144.

Klaenhammer, T.R., and E.G. Kleeman. 1981. Growth characteristics, bile sensitivity, and freeze damage in colonial variants of *Lactobacillus acidophilus*,. Appl. Environ. Microbiol. 41: 1461-1467.

Klaver, F.A.M., and R. Van der Meer. 1993. The assumed assimilation of cholesterol by lactobacilli and *Bifidobacterium bifidum* is due to their bile salt-deconjugating activity. Appl. Environ. Microbiol. 59: 1120-1124.

Kole, M., and I. Altosaar. 1984. Bovine bile resistance increases *Leuconostoc oenos* 44.40 viability upon lyophilization. Appl. Environ. Microbiol. 47: 1150-1153.

Kopeloff, N. 1934. Dissociation and filtration of *Lactobacillus acidopilus*. J. Infect. Dis. 55: 368-379.

Kosikowski, F. 1977. Cheese and fermented milk foods, 2nd ed. Edwards Brothers, Ann Arbor, MI, U.S.A.

Lauer, E., and O. Kandler. 1980. *Lactobacillus gasseri* sp. nov., a new species of the subgenus Thermobacterium. Zbl. Bakt., I. Abt. Orig. C 1, 75-78.

Leer, R.J., H. Christiaens, W. Verstraete, L. Peters, M. Posno, and P.H. Pouwels. 1993. Gene disruption in *Lactobacillus plantarum* strain 80 by site-specific recombination: isolation of a mutant strain deficient in conjugated bile salt hydrolase activity. Mol. Gen. Genet. 239: 269-272.

Leppik, R.A. 1989. The genetics of bile acid degradation in *Pseudomonas* spp.: Location and cloning of catabolic genes. J. Gen. Microbiol. 135: 1989-1996.

Lidbeck, A., J.-Å. Gustafsson, and C.E. Nord. 1987. Impact of *Lactobacillus acidophilus* supplements on the human oropharyngeal and intestinal microflora. Scand. J. Infect. Dis. 19: 531-537.

Ling, W.H., R. Korpela, H. Mykkänen, S. Salminen, and O. Hänninen. 1994. *Lactobacillus* strain GG supplementation decreases colonic hydrolytic and reductive enzyme activities in healthy female adults. J. Nutr. 124: 18-23.

Ma, D., D.N. Cook, M. Alberti, N.G. Pon, H. Nikaido, and J. E. Hearst. 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Molecular Microbiology 16: 45-55.

Macrina, F.L., D.J. Kopecko, K.R. Jones, D.J. Ayers, and S.M. McCowen. 1978. A multiple plasmid-containing *Escherichia coli* strain: Convenient source of size reference plasmid molecules. Plasmid 1: 417-420.

Mallory, A., F. Kern, Jr., J. Smith, and D. Savage. 1973. Patterns of bile acids and microflora in the human small intestine. I. Bile acids. Gastroenterology 64: 26-33.

Marshall, V.M. 1991. Gut-derived organisms for milk fermentations, p.548-553. In Biotechnology group meeting. Probiotics - fact or fiction? J. Chem. Tech. Biotechnol. 51: 539-570.

Marteau, P., M. Minekus, R. Havenaar, and J.H.J. Huis in't Veld. 1993. Study of the delivery of ingested microorganisms to target sites beyond the stomach using an in vitro model -a pharmacokinetical approach for probiotics. Gastroenterology 104: A546.

McFarland, L.V., and P. Bernasconi. 1993. *Saccharomyces boulardii*:: A review of an innovative therapeutic agent. Microbial Ecology in Health and Disease 6: 157-171.

Merck Index. 1983. Windholz, M. (ed.). Merck & Co., Inc., Rahway, N.J., U.S.A.

Meurman, J.H., H. Antila, and S. Salminen. 1994. Recovery of *Lactobacillus* strain GG (ATCC 53103) from saliva of healthy volunteers after consumption of yoghurt prepared with the bacterium. Microbial Ecology in Health and Disease. 7: 295-298.

Minekus, M., P. Marteau, R. Havenaar, and J.H.J. Huis in't Veld. 1993. Development of a computer controlled *in vitro* model of the gastriontestinal tract. Gastroenterology 104: A553.

Mital, B.K., and S.K. Garg. 1992. Acidophilus milk products: manufacture and therapeutics. Food Rev. Int. 8: 347-389.

Mitsuoka, T. 1978. Intestinal bacteria nad health. Harcourt Brace Jovanovich Japan, Inc., Tokyo, Japan.

Molin, G., B. Jeppson, M.-L. Johansson, S. Ahrné, S. Nobaek, M. Ståhl, and S. Bengmark. 1993. Numerical taxonomy of *Lactobacillus* spp. associated with healthy and diseased mucosa of the human intestines. J. Appl. Bacteriol. 74: 314-323.

Molly, K., M. Vande Woestyne, and W, Verstraete. 1993. Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. Appl. Microbiol. Biotechnol. 39: 254-258.

Noh, D. O., and S. E. Gilliland. 1993. Influence of bile on cellular integrity and β-galactosidase activity of *Lactobacillus acidophilus*. J. Dairy Sci. 76: 1253-1259.

Oksanen, P.J., S. Salminen, M, Saxelin, P. Hämäläinen, A. Ihantola-Vormisto, L. Muurasniemi-Isoviita, S. Nikkari, T. Oksanen, I. Pörsti, E. Salminen, S. Siitonen, H. Stuckey, A. Toppila, and H. Vapaatalo. 1990. Prevention of traveller's diarrhoea by *Lactobacillus* GG. Ann. Med. 22: 53-56.

Orla-Jensen, S. 1919. The lactic acid bacteria. Copenhagen: Andr. Fred. Host and Son.

Orrhage, K., B. Brismar, and C.E. Nord. 1994. Effect of supplements with *Bifidobacterium longum* and *Lactobacillus acidophilus* on the intestinal microbiota during administration of clindamycin. Microbial Ecology in Health and Disease 7: 17-25.

O'Sullivan, D.J., and T.R. Klaenhammer. 1993. Rapid mini-prep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. Appl. Environ. Microbiol. 59: 2730-2733.

O'Sullivan, M.G., G. Thornton, G.C. O'Sullivan, and J.K. Collins. 1992. Probiotic bacteria: myth or reality? Trends Food Sci. and Technol. 31: 309-314.

Pot, B., W. Ludwig, K. Kersters, and K.-H. Schleifer. 1994. Taxonomy of lactic acid bacteria, p. 14-90. In Bacteriocins of lactic acid bacteria. L. De Vuyst and E.J. Vandamme (ed.). Blackie Academic & Professional, Great Britain.

Rahe, A.H. 1915. A study of the so-called implantation of *Bacillus bulgaricus*. J. Infect. Dis. 16: 210-220.

Roberts, F.J., I.W. Geere, and A. Goldman. 1991. A three-year study of positive blood cultures, with emphasis on prognosis. Rev. Infect. Dis. 13: 34-46.

Robins-Browne, R.M., F.F. Path, and M.M. Levine. 1981. The fate of ingested lactobacilli in the proximal intestine. Am. J. Clin. Nutr. 34:514-519.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Savoie, L., and S.F. Gauthier. 1986. Dialysis cell for the in vitro measurement of protein digestibility. J. Food Sci. 51: 494-498.

Saxelin, M., S. Elo, S. Salminen, and H. Vapaatalo. 1991. Dose response colonisation of faeces after oral administration of *Lactobacillus casei* strain GG. Microbial Ecology in Health and Disease 4: 209-219.

Sharpe, M.E. 1979. Lactic acid bacteria in the dairy industry. J. Soc. Dairy Technol. 32: 9-18.

Shaw, B.G. , and C.D. Harding. 1985. Atypical lactobacilli from vacuum-packaged meats: comparison by DNA hybridization, cell composition and biochemical tests with a description of *Lactobacillus carnis* sp. nov. System. Appl. Microbiol. 6: 291-297.

Shimada, K., K.S. Bricknell, and S.M. Finegold. 1969. Deconjugation of bile acids by intestinal bacteria: Review of literature and additional studies. J. Infect. Dis. 119: 273-281.

Silva, M., N.V. Jacobus, C. Deneke, and S.L. Gorbach. 1987. Antimicrobial substance from a human *Lactobacillus* strain. Antimicrob. Agents and Chemother. 31: 1231-1233.

Sjövall, J. 1959. On the concentration of bile acids in the human intestine during absorption. Acta Physiol. Scand. 46: 339-345.

Spallholz, J.E. 1989. Nutriton chemistry & biology. Prentice Hall, Englewood Cliffs, New Jersey 07632, U. S. A.

Stryer, L. 1988. Biochemistry, 3rd ed. Freeman and Company, New York, U.S.A., p. 559.

Sturkie, P.D. 1981. Basic Physiology. Springer-Verlag, New York, U. S. A.

Sung, J.Y., E.A. Shaffer, and J.W. Costerton. 1993. Antibacterial activity of bile salts against common biliary pathogens. Digestive Diseases and Sciences 38: 2104-2112.

Tannock, G.W., M.P. Dashkevicz, and S.D. Feighner. 1989. Lactobacilli and bile salt hydrolase in the murine intestinal tract. Appl. Environ. Microbiol. 55: 1848-1851.

Van der Meer, R., D.S.M.L. Termont, and H.T. De Vries. 1991. Differential effects of calcium ions and calcium phosphate on cytotoxicity of bile acids. Am. J. Physiol. 260: G142-G147.

Van Eldere, J., J. Robben, G. De Paww, R. Merckx, and H. Eyssen. 1988. Isolation and identification of intestinal steroid-desulfating bacteria from rats and humans. Appl. Environ. Microbiol. 54: 2112-2117.

Wayne, L.G. 1994. Actions of the Judicial Comission of the International Committee on Systematic Bacteriology on requests on opinions published between January 1985 and July 1993. Int. J. Syst. Bacteriol. 44:177-178.

Zoppi, G., V. Balsamo, A. Deganello, G. Iacomo, F. Saccomani, and G. Benoni. 1982. Oral bacteriotherapy in clinical practice. II. The use of different preparations in the treatment of acute diarrhoea. Eur. J. Pediatr. 139: 22-24.